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OBITUARY NOTICES.

EDWARD STAFFORD EDIE (1879–1927).

EDIE was born in 1879 at Cornceres, Anstruther in Fife. After early education in Anstruther he went to Edinburgh where he attended George Watson's College and then the University of Edinburgh. At the University he took courses which led to his obtaining an M.A. and B.Sc. in Chemistry.

His first work after graduation was an investigation into the conditions of production of Gum Acacia. For that purpose he worked for two seasons south of Khartoum in the Sudan. This work indicates that he became interested in Biological Chemistry at a time when the number of workers at this border-line subject were few in number.

He next went to Liverpool where he worked with the late Professor Benjamin Moore. At that time the Biochemical Department at Liverpool had been recently established and it was a very active centre for research. Edie was one of those who took part in most of the projects which were initiated during that period. The scope of these activities can be judged by the numerous papers on which his name appeared as author or joint author. Many of these papers are to be found in the early volumes of this *Journal* commencing with volume 1. Amongst other subjects he studied the production of glycosuria by excess of carbon dioxide and by other agents. He also assisted in the study of alkali output in the urine, the nutrition of marine organisms, the staining of cotton by methylene blue and the preparation of lecithin.

In 1912 he was appointed Lecturer in Biochemistry in the Department of Physiology at Aberdeen where he worked until 1921. During that period his original work consisted in the study of enzyme action.

In 1921 he obtained his degree of Doctor of Science from the University of Edinburgh for a thesis entitled Biochemical Researches.

Edie was appointed the first Professor of Biochemistry at the University of Cape Town in 1921. This meant the organisation of a Department, the acquirement of equipment and finally the erection of a laboratory. He undertook these duties with conspicuous ability and during his six years in Cape Town he established a high reputation amongst his colleagues. Whilst on long leave in this country he died suddenly in December 1927. Thus he was unable to see the opening of the new buildings, the construction of which had occupied so much of his time and thought.

In 1914 Edie married the younger daughter of the late Rev. John Galloway of the United Free Church of Kintore, Aberdeenshire, and he is survived by his widow, a son and a daughter.

Those who worked with him were always impressed by his unfailing good nature and his willingness to help in whatever project was being undertaken. A slight deformity, due to infantile paralysis, prevented him from taking part in some outdoor activities but it did not prevent him from acquiring a high degree of experimental skill.

H. E. R.

ERNEST HENRY STARLING (1866–1927).

Ernest Henry Starling died suddenly on board the *Ariquaing* on May 2nd of last year, as the ship was approaching Kingston, Jamaica, and was buried on the following day at Halfway Tree Parish Church, near Kingston. His death deprives us of one of the greatest figures in a generation of men of individuality and striking personality. Starling's influence on physiology was great, not only because he was himself a great physiologist with a wide grasp of his subject, nor because of the many ramifications of his work, but very largely on account of his potent influence over others.

Destined by his outstanding and thrilling personality to dominate and to lead, and endowed with tremendous energy, Starling enjoyed a career which, though brilliant from its commencement, was probably even more so towards its close. His natural craving for exact knowledge on the one hand, and his early and growing belief in the power with which physiological knowledge might be applied to the control and conquest of suffering on the other, attracted him, while still at Guy's Hospital as a medical student, to the study of physiology. Not the least of his gifts was a lively, almost kaleidoscopic, imagination, kept within bounds and tempered by his love for truth.

His training in its first stages, was essentially that of a medical student; he was greatly fascinated by chemistry, as presented by his teacher Dr Debus, to whose enthusiasm Starling readily responded. This taste for chemistry remained with him and no doubt greatly influenced his subsequent life. During his clinical work it became clear to him that the fruits of mere observation had all been gathered centuries ago and that only by properly controlled experiment could the key be found to the fascinating puzzles of physiology and the urgent needs of medicine. "Science," he said, "has but one language, that of quantity, and but one argument, that of experiment."

By working at Heidelberg in Kühne's laboratory while still a student, and subsequently with Heidehain at Breslau, he became more acquainted with the methods of exact scientific investigation. In those days there were no well-paid research posts into which young men could at a wish subside, and during his subsequent career as lecturer on physiology (and Grocers' Research Scholar), at Guy's Hospital from 1889–1899, as Jodrell Professor of Physiology at University College, London, until 1923, and during his last years as Foulerton Research Professor of the Royal Society, as he worked strenuously, the conviction of the value of experiment steadily grew upon

him. He was elected a Fellow of the Royal Society in 1899, and, besides many other honours including five honorary degrees, was awarded the Royal medal in 1913, and the C.M.G. for his war services.

Starling's sympathies were of the widest, and his knowledge of the most extensive: he believed firmly in the unity of science. It was in his relations to biology that he flung his net widest, but discoveries in the exact sciences were eagerly adopted by him and given physiological bearings. Although at no time in his career could Starling have been described as being familiar at first hand with methods of chemical investigation, yet so true was his instinct for the things that mattered that he was ever eager to assimilate and utilise chemical knowledge and by his teaching to disseminate those advances in physical and organic chemistry which in his own time played such a large part in the development of the scientific aspects of physiology. His constant desire to be able to expound well-connected chemical histories of intermediary metabolism was always apparent in his lectures and in his text-book; work like that of Knoop on β-oxidation was eagerly seized upon, and discoveries like those of insulin or thyroxine at once excited his admiration and enthusiasm. His investigations on the formation of lymph, for example, were well ahead of the physico-chemical views of the period, and have suffered little modification since. The greatest work of biochemical significance with which Starling was associated was that done jointly with his well-loved colleague and brother-in-law, the late Sir William Bayliss, which led to the discovery of secretin, and provided the first really well-documented example of the regulation of bodily function by chemical substances. The name hormone was indeed introduced by Bayliss and Starling as the outcome of this work, and the chemical correlation of the functions of the body formed the subject of their Croonian Lecture to the Royal Society in 1904.

During his tense career Starling, by his genius for constructive work, created two new physiological laboratories. One, at Guy's Hospital, was barely completed and equipped as the best at the time in London, when he moved to University College and planned on a yet more ambitious scale to create an Institute of the Medical Sciences. The first stage of this to be realised was the present Institute of Physiology opened in 1909; this was followed by an adjoining Pharmacology department, and afterwards, owing to a generous gift from the Rockefeller Foundation, the ideal scheme was happily completed by the construction of a department of Anatomy and Embryology, with a well-filled common central library for the Medical Sciences.

Always virile, Starling loved a fight in a worthy cause, and during the war was with difficulty prevented from manœuvring his way into a combatant unit. Instead he served in the R.A.M.C., chiefly in the gas warfare services, both at Millbank, where for a time he was director of the research on gas defence problems, and later at Salonika as Chemical Adviser. During his stay at Millbank he and Lieut. (afterwards Colonel) E. H. Harrison accom-

plished great things in the face of considerable difficulties. He resigned his commission in 1917 and was made Chairman of the Royal Society Food Committee and Scientific Adviser to the Ministry of Food. He retained links with the gas services, however, and one of the writer's most pleasant memories of his association with Starling is that of a joint visit to Italy in the spring of 1918 to consult with the Italian Military authorities on the choice of a respirator—a mission which Starling carried out to the complete satisfaction of the Italian forces and of our own Gas Warfare authorities.

Starling may be described, I think, as the last of the great masters of experimental physiology, as the last among physiologists able himself to grasp and expound all branches of the subject, including a great deal of very sound biochemistry. Starling became famous certainly for his researches, for the intensity and reality of his teaching, for his fine and accurate writing, of which the greatest example was his world-famous *Principles of Human Physiology*, but will be remembered longest by his friends and pupils by his personality. Who could withstand that frank and fearless eye, when it lit up with an eagerness that was boyish to the last, or that engaging smile of his? His pupils and colleagues loved him, and he gave them his best. By nature he was impetuous, but seemed singularly able to inhibit all but generous impulses, though he was impatient of fools, officials and rogues; he loved life and young people, and can be said never to have grown old.

In 1910 he had a serious breakdown from overwork, but after a trip to Japan returned full of his old energy, and in less than two years had produced his *Principles of Human Physiology*. On June 10th, 1920, after considerable suffering, he underwent a severe operation followed by tedious sequelae: once more he rallied, and when in 1923 he retired from the Jodrell Chair his friends hoped and believed that he would be able to devote many unhampered years to research work. Though this was not to be, he had the satisfaction of working almost to the last, and of going down abruptly, with all flags flying and we know that was what he wished.

His devoted wife, who helped him greatly in his work, was the widow of his colleague, Wooldridge; she survived him by only a few months, and died on January 24th, 1928.

C. L. E.

LXXIX. A MODIFIED METHOD FOR THE DETERMINATION OF HIPPURIC ACID AND FREE BENZOIC ACID IN THE URINE OF CATTLE.

By FREDERICK JOHN WARTH and
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(*Received February 28th, 1928.*)

Hippuric acid is usually estimated by hydrolysing the substance and determining one or other of the constituents. If hydrolysis is effected with acid the resulting amino-acid is determined. If alkali is used for the hydrolysis the resulting benzoic acid is determined. For work on which we are engaged, the existing methods have not been found convenient. Our experience with procedures in which acid hydrolysis is employed has been unsatisfactory generally. In all cases the hydrolysed liquid requires lengthy manipulation to prepare it for the determination. We have also found the formaldehyde titration method unreliable with our urine samples, although we introduced a refinement by titrating with the aid of a comparator. The best known methods, in which alkaline hydrolysis is employed, are those of Folin and Flanders [1912] and of Kingsbury and Swanson [1921]. We have found the method of Folin and Flanders satisfactory for small amounts of hippuric acid but not so good when considerable quantities have to be determined. We have tested the method of alkaline hydrolysis recommended by Kingsbury and Swanson and find it entirely satisfactory. These authors boil 50 cc. of liquid with 7·5 g. NaOH in a Kjeldahl flask for half an hour, reducing the volume to 25 cc. during this time. We find that this amount of alkali is just sufficient to ensure hydrolysis. For safety we prefer to boil down twice.

Folin and Flanders, and Kingsbury and Swanson employ similar methods for determining the benzoic acid liberated by hydrolysis. The liquid is oxidised with nitric acid under a reflux condenser to destroy colouring matters and emulsifying substances. The benzoic acid is then completely extracted by shaking out repeatedly with chloroform and the chloroform solution is washed and titrated. We have observed the emulsifying action of the raw hydrolysate upon chloroform and agree with the above-mentioned authors that clean and complete extraction is impossible without preliminary oxidation. The time and labour involved in the oxidation are, however, a serious drawback to the

process. Therefore, as a possible means of avoiding this lengthy operation entirely, the senior author proposed the following new procedure.

After alkaline hydrolysis the liquid is acidified, made to a definite volume and shaken with a definite volume of immiscible non-volatile solvent. Kerosene was proposed for this purpose. The benzoic acid in the solvent is determined by titrating an aliquot part. The benzoic acid remaining in the aqueous layer is calculated from previously determined partition coefficients. By this means the necessity for complete extraction is avoided. We have tested this procedure and have obtained entirely satisfactory results with it. The experimental work falls under three heads.

1. A study of the partition coefficients of benzoic acid.
2. The determination of hippuric acid in urine.
3. The determination of free benzoic acid in urine.

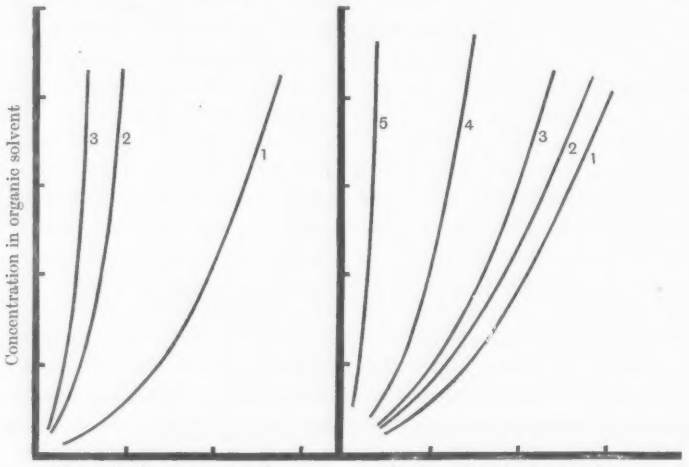

Chart 1.

Organic solvent variable
1. Kerosene.
2. Toluene.
3. Chloroform.
Aqueous layer constant
Water.

Chart 2.

Organic solvent constant
Kerosene.
Aqueous layer variable
1. Water. 2. 1 % HCl.
3. 3 % NaCl in 1 % HCl.
4. 10 % NaCl in 1 % HCl.
5. Saturated NaCl in 1 % HCl.

PARTITION COEFFICIENTS OF BENZOIC ACID.

1. *Partition between water and different solvents.* Tests were made with kerosene, toluene and chloroform. The results are shown in Chart 1. The chart brings out two points.

(*a*) The partition of benzoic acid between water and any of these solvents is a function of the concentration. The proportion of benzoic acid passing into the organic solvent increases rapidly with the concentration.

(*b*) Chloroform and toluene are very efficient solvents for benzoic acid, chloroform being slightly superior to toluene. Kerosene has a low efficiency, especially when the concentration of benzoic acid is low.

2. *The effect of mineral acids and salts on the partition of benzoic acid.* As salts and mineral acids must always be present after hydrolysis, it was necessary to determine their effect on the partition. Typical experimental results are given in Chart 2. In these tests the organic solvent (kerosene) was the constant and the aqueous layer the variable. The sodium chloride solutions all contained 1 % HCl. The chart shows that the efficiency of extraction increases regularly with increasing concentration of sodium chloride. The increase is so great that kerosene with saturated salt solution becomes as effective as chloroform with water. This property of sodium chloride is of great practical value and has been made use of by us in our method for determining benzoic acid. Presumably other salts exert a similar influence. For example $(NH_4)_2SO_4$ has been used for the same purpose by Delprat and Whipple [1921]. We have confined our tests to sodium chloride because its solubility is almost unaffected by small changes in temperature, a property which makes it particularly suitable for our purpose.

3. *Effect of temperature on the partition of benzoic acid.* To observe the effect of temperature, the partitions at 20° and 30° were compared. The temperature factor was considerable in the case of water without mineral acid and salt. It became less marked as the salinity increased. In every case, the partition was less favourable at the higher temperature. The results were perfectly regular, but it is only necessary to quote the figures obtained with saturated sodium chloride, since, for practical purposes, only saturated solutions are used by us now. The figures are included in Table I.

4. *Standard partition coefficients with saturated sodium chloride in* 1 % HCl. The preliminary experiments having shown that saturated sodium chloride was the best working medium, more exact determinations, covering a wider range, have been carried out with saturated solutions. The results are shown in Table I, which, in graphic form, is our working basis for the determination of benzoic acid.

Table I. *Standard partition determinations.*

Kerosene and saturated NaCl in 1 % HCl 20°		Kerosene and saturated NaCl in 1 % HCl 30°		Kerosene and saturated NaCl in 2 % HCl 30°		Toluene and saturated NaCl in 1 % HCl 30°	
K	W	K	W	K	W	T	W
3·40	0·43	3·20	0·47	3·30	0·49	7·8	0·24
7·80	0·68	7·50	0·73	7·45	0·81	16·2	0·35
17·50	1·01	16·75	1·14	16·70	1·27	33·2	0·54
27·40	1·30	26·40	1·48	26·35	1·64	51·0	0·56
42·70	1·66	41·50	1·88	41·30	2·11	68·4	0·67
58·30	1·96	57·00	2·20	56·80	2·46	—	

The figures in this table represent corresponding equilibrium concentrations in the two phases. The concentration is expressed as cc. $N/7$ benzoic acid per 100 cc. liquid.

The points of practical importance in these figures are as follows.

(*a*) The temperature factor is small. It is unnecessary to use a thermostat as long as the laboratory temperature lies between 20° and 30°. The actual temperature is noted and the partition for that temperature is read off from a large scale graph.

(*b*) The concentration of mineral acid has a perceptible but not an appreciable effect.

(*c*) The figures for kerosene are so favourable that there is generally no advantage in using toluene.

The determination of hippuric acid.

The steps in the new procedure are the following.

1. *Hydrolysis with NaOH.* This has been described above.

2. *Preparation of the solution.* The liquid in the Kjeldahl flask is acidified and then made just alkaline. Solid sodium chloride is added in excess and shaken. The liquid is transferred to a 110 cc. flask, saturated sodium chloride being used for rinsing. The liquid is made up to 110 cc. with saturated sodium chloride. 100 cc. are pipetted out into a shaking bottle and 10 cc. of 10 % HCl saturated with sodium chloride are added. Benzoic acid is thus liberated in a known volume of saturated sodium chloride solution. Some solid sodium chloride and a definite volume of kerosene (or toluene) are added and the bottle is shaken at room temperature. The entire contents of the bottle are poured into a separating funnel and allowed to stand until the two liquids have separated. Kerosene and toluene are found to separate better than chloroform. The aqueous layer is run out and the kerosene is transferred to a clean, dry beaker and allowed to stand for some time. All the remaining water separates out on the sides of the vessel and a perfectly clean kerosene solution of benzoic acid is obtained. We have titrated many such solutions and find that there is no contamination with mineral acid. Washing is unnecessary, but it is advisable to filter the kerosene through a dry filter even if it appears to be quite clear. An aliquot part of the filtrate is titrated.

3. *Titration of kerosene and toluene solutions of benzoic acid.* An equal volume of alcohol is added to the liquid in an Erlenmeyer flask. The contents are mixed with a rotary motion and titrated with aqueous standard alkali. The toluene and alcohol form a clear homogeneous mixture which is very easily titrated. The kerosene and alcohol do not mix completely, a milky emulsion being obtained. Theoretically such a two-phase liquid might not be expected to titrate either rapidly or completely, but practical experience with many standard solutions of benzoic acid shows that the method always yields quantitative results. It may be mentioned here that we have abandoned the use of alcoholic potash for chloroform titrations also, and have adopted the above procedure instead. With chloroform, however, more alcohol is required and the titration is slower.

Examples.

1. 0·801 g. pure hippuric acid, treated as described, required 26·90 cc. $N/7$ alkali.

The graph shows that the aqueous layer must contain 1·40 cc. $N/7$ benzoic acid per 100 cc. or 1·54 cc. in 110 cc. Hence 0·7999 g. hippuric acid is found, which agrees very well with the quantity taken.

2. *Tests with urine.* The following figures from four separate tests on the same sample of urine show that duplicates give concordant results. 50 cc. of the kerosene extract gave titrations of 7·25, 7·25, 7·30 and 7·25 cc., respectively.

It is more difficult to prove that the method does not introduce systematic errors when applied to urine analysis. As pure hippuric acid yields theoretical results, a systematic error in urine analysis can only be due to urinary constituents. These constituents may, conceivably, cause errors in two ways. Firstly, the partition of benzoic acid may be altered. Secondly, titratable impurities may be extracted by the kerosene. The first error can be checked with certainty by carrying out duplicate analyses with different quantities of urine. The second error will be revealed in the same way unless the impurity happens to have exactly the same partition coefficients as benzoic acid, which is a very unlikely circumstance.

Accordingly four lots of 50 cc. and four lots of 25 cc. urine were submitted to the process. The same amount of alkali was used in all cases and the liquids were all made up to the same volume. The following results were obtained.

Table II. *Hippuric acid found (g. per 100 cc. urine).*

	50 cc. tests	25 cc. tests
1.	1·285	1·287
2.	1·285	1·287
3.	1·304	1·298
4.	1·285	1·298

The figures are a fair representation of the accuracy and reliability of the method and indicate that there is no appreciable systematic error in the determinations.

The determination of free benzoic acid in urine.

In determining small quantities of free benzoic acid by any of the existing methods, the question arises whether the titration obtained is due to benzoic acid or not. By our partition method some indirect information can be gained on this point. It should be noted here that whenever kerosene (or toluene) extraction is spoken of in the sequel it refers to the extraction of a liquid saturated with sodium chloride and containing 1 % HCl. We have frequently carried out kerosene extractions of unhydrolysed urine and have obtained perceptible amounts of acidity from perfectly fresh urine. The quantities are very small, ranging generally between 3 and 5 drops of $N/7$ acid in 50 cc. urine. Is this benzoic acid? Duplicate extractions with kerosene and toluene were

carried out. Toluene was found to indicate a higher percentage acidity. Therefore either our partition coefficients have broken down at this extreme range, or the substance extracted is not benzoic acid. To investigate this discrepancy, minute quantities of benzoic acid were added to urine, which was then extracted as usual. Blank determinations on the urine alone were made simultaneously. The same sample of urine was tested with both solvents. The results obtained are given in Table III.

Table III.

	cc. $N/7$ benzoic acid added to urine	cc. $N/7$ acidity found in urine	Excess over blank
Kerosene extraction	0	0·58	0
	1·35	1·83	1·25
	2·70	3·19	2·61
Toluene extraction	0	0·89	0
	1·00	2·04	1·15
	2·00	3·01	2·12

The figures show that very small amounts of added benzoic acid can be accounted for satisfactorily. This proves that the standard partition coefficients must apply with a fair degree of accuracy to urine. We are inclined to conclude from this experiment that the blank titration with toluene does not represent an equivalent amount of free benzoic acid, but is, at least partly, due to some other substance which is more soluble in toluene than in kerosene. Chloroform is liable to take up considerably more titratable impurities than toluene. Extractions were carried out with the three solvents on a sample of fresh urine. In each case 80 cc. of urine and 60 cc. of solvent were used. After separation, 50 cc. of the solvent were titrated (Table IV).

Table IV.

	Acidity in 50 cc. solvent	Acidity in 60 cc. solvent	Computed free benzoic acid in urine
Chloroform test	1·80	2·16	>2·16
Toluene test	0·40	0·48	0·50
Kerosene test	0·15	0·18	0·20

Judging by the results of Table IV it seems certain that the titration obtained with chloroform in this test cannot be due to benzoic acid. Our conclusion is that kerosene is probably the safest solvent to use when free benzoic acid has to be determined.

Concerning the determination of free benzoic acid by means of kerosene, the figures of Table III show that 1·3 cc. $N/7$ free benzoic acid in 100 cc. of urine can be easily determined by this method.

Finally a word must be said regarding the relative merits of the three solvents. Kerosene has many points in its favour. It is, for our purpose, non-volatile; it separates most easily and most completely from the aqueous layer; it takes up a minimum amount of titratable impurity; its low cost is a further point in its favour. The disadvantages of kerosene are that it is indefinite in

composition and that its solvent power for benzoic acid is low. Toluene has a greater solvent power but, to set off this advantage, it may take up more titratable impurity; it is also somewhat volatile. Chloroform is too volatile to be of use for our method.

SUMMARY.

1. A modified method for the determination of hippuric acid is described. The feature of the method consists in the estimation of the liberated benzoic acid with the aid of partition coefficients. A tedious operation of oxidation is eliminated by this means.

2. The method, when applied to unhydrolysed urine, gives some indication that titratable impurities are likely to be determined with the free benzoic acid.

3. Kerosene possesses certain advantages as a solvent in the determination of benzoic acid.

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LXXX. OBSERVATIONS ON PHOSPHATES IN BLOOD AND ON THE URINARY EXCRETION OF PHOSPHATES.

By REGINALD THOMAS BRAIN,
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(*Received February 29th, 1928.*)

It has been suggested from time to time (*a*) that "inorganic" phosphate in the blood or plasma is present in some unusual form (*i.e.* not as true ionic phosphate), (*b*) that the excretion of phosphate in the urine is controlled by factors other than the level of "inorganic" phosphate in the blood.

We bring together in this paper a number of observations we have made on these two, and on other related questions. We shall deal, in particular, with the interesting conclusion of Eichholtz, Robison and Brull [1925] that it is not the inorganic phosphate but the acid-soluble organic phosphorus of the plasma that is the normal source of part, if not all, of the inorganic phosphate of the urine, the organic phosphorus undergoing hydrolysis in the cells of the kidney under the action of kidney phosphatase.

Fluid reaching the kidney.

Phosphate concentration in the plasma.

Mayrs [1922], Wu [1922] and Cushny [1926] have emphasised that it is the concentration of a substance in the *plasma* and not that in the whole blood which is of major importance in relation to urinary excretion. With regard to inorganic phosphate it has been variously stated that the normal corpuscular content of inorganic phosphate is greater than [Bloor, 1918; McKellips, de Young and Bloor, 1921; Jones and Nye, 1921], equal to [Zucker and Gutman, 1922; Wigglesworth and Woodrow, 1923], or less than [Kerr, 1926; Underhill, 1923] that in the plasma. Richter-Quittner [1921] and Buell [1923] go so far as to conclude that there is no inorganic phosphate in the corpuscles.

We have investigated this question by two methods.

First method. Samples of blood[1] were collected into paraffined centrifuge tubes over ice, and the separation of the plasma and precipitation of the

[1] Human blood has been used throughout our work, unless otherwise stated.

proteins carried out with the utmost celerity. Samples of blood drawn at the same time, but prevented from clotting by heparin and by oxalate were also dealt with similarly. Briggs's method was used for inorganic phosphate determinations. Corpuscle phosphate was calculated from these determinations and the haematocrit reading. Results are in Table I.

Table I. *Inorganic phosphate distribution between plasma and corpuscles, P mg. per 100 cc.*

	Cooled blood, no anticoagulant				Same blood not cooled							
					Oxalate 0·2 %				Heparin 0·02 %			
Exp.* No.	Blood	Plasma	Corps.	$\frac{\text{Plasma}}{\text{Corps.}}$	Blood	Plasma	Corps.	$\frac{\text{Plasma}}{\text{Corps.}}$	Blood	Plasma	Corps.	$\frac{\text{Plasma}}{\text{Corps.}}$
1	3·1	3·3	2·9	1·14	2·7	3·1	2·2	1·41	2·8	2·9	2·7	1·07
2	3·0	3·5	2·4	1·46	2·7	3·2	2·0	1·60	2·9	3·6	2·0	1·80
3	5·0	6·1	3·6	1·69	4·7	5·7	3·3	1·73	4·7	6·1	3·1	1·97
4	3·5	4·3	2·4	1·79	3·5	3·9	2·8	1·39	3·5	4·1	2·5	1·64
5	2·3	2·8	1·5	1·86	2·2	2·6	1·6	1·63	2·2	2·8	1·5	1·87
6	3·6	4·7	1·9	2·47	3·6	4·3	2·5	1·76	3·7	4·5	2·4	1·87
Mean	3·4	4·1	2·4	1·71	3·2	3·8	2·4	1·58	3·3	4·0	2·4	1·67

* Experiments in order of increasing $\frac{\text{plasma}}{\text{corps.}}$ ratio.

It will be seen that for both blood and plasma the results are consistently just a little higher when no anticoagulant is used than in the oxalated specimens, though the difference is not very great. Whatever the method employed to prevent clotting, free phosphate is invariably found within the corpuscles albeit in distinctly lower concentration than in the plasma. If the corpuscles are washed with isotonic sodium chloride solution, appreciable quantities of inorganic phosphate may be found in the washings, at the expense of that in the corpuscles. This probably accounts for the findings of Buell that little or no free phosphate is present in the corpuscles, since she found it necessary to wash the corpuscles in some cases as many as four times before analysis.

We have carried out a large number of determinations during the past two years upon samples of oxalated blood from normal subjects and from patients with nephritis, working without undue delay, but with no special celerity. On the average, the plasma values are somewhat lower, and the corpuscle values slightly higher than in Table I. Average values from 85 determinations on normal blood and 36 on blood from patients with nephritis are:

	Inorg. P mg. per 100 cc.			Plasma
	Blood	Plasma	Corps.	$\frac{\text{Plasma}}{\text{Corps.}}$
Normal subjects or convalescent patients	3·9	4·5	3·3	1·36
Patients with nephritis	6·5	7·3	4·7	1·55

The plasma value for inorganic phosphate in nearly all instances is higher than the corpuscle value, whether the blood phosphate was rising, falling or constant.

The results are shown graphically in Figs. 1 and 2. For normal blood, the points lie on a curve $p = 1{\cdot}3\,b - 0{\cdot}7$, where p is the plasma and b the blood phosphate value. This equation has been found useful for obtaining probable plasma values from the whole blood values of other workers.

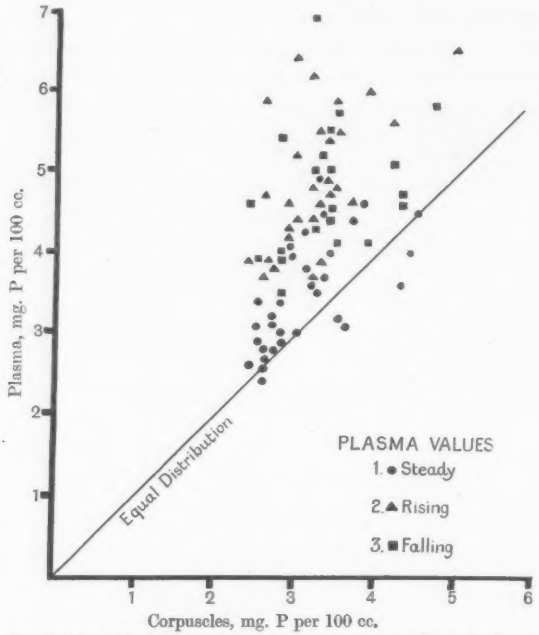

Fig. 1. The distribution of inorganic phosphate between corpuscles and plasma in different phases of varying plasma concentration.

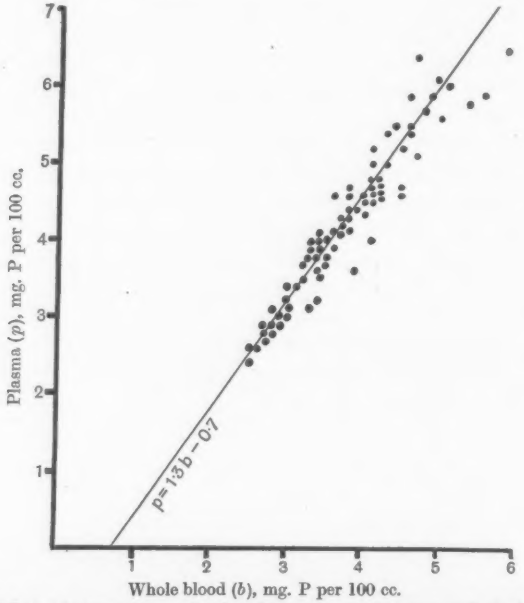

Fig. 2. Relation between inorganic phosphate content of plasma and of whole blood, in normal subjects.

Second method. Foetal blood was centrifuged in umbilical cords. By this method the blood remains in endothelium-lined blood-vessels, and as nearly normal in environment as possible, until the plasma has been separated; the red cells therefore are more likely to escape injury during centrifugation.

The cord is obtained as early as possible after the birth of the child; a portion, engorged with blood, is secured between ligatures, the blood being mixed by gently squeezing the cord. A third ligature is then introduced between the other two, and a sample of whole blood withdrawn from one of the two portions of cord and run into a tube containing just sufficient solid oxalate to prevent clotting. The remainder of the cord is suspended in liquid paraffin in a centrifuge tube and spun, the plasma removed by a fine needle and syringe and run on to oxalate. An inorganic phosphate determination is then made on both plasma and whole blood. A small portion of unoxalated blood, prevented from clotting by heparin, is used for the haematocrit determination. The results of four such experiments are shown in Table II.

Table II. *Blood centrifuged in the vein. P mg. per 100 cc.*

Exp. No.	Inorg. P concentration in Blood	Plasma	Corps.	Plasma / Corps.
1	4·4	5·8	2·2	2·64
2	3·1	4·7	1·7	2·76
(2 *a**	3·1	4·4	2·1	2·10)
3	4·6	6·1	2·7	2·26
4	5·1	6·7	3·2	2·09
(4 *a**	5·1	5·8	4·0	1·45)
Average for 1, 2, 3 and 4				2·44

* Specimen of whole blood from same cord centrifuged *after* removing from vein and oxalating.

The distribution of inorganic phosphate between the plasma and corpuscles in foetal blood centrifuged in the vein differs, therefore, from that in adult blood centrifuged, however speedily, *in vitro*. There is relatively much more phosphate in the plasma in the former case than in the latter.

The foregoing results are not altogether surprising in view of two interrelated factors which will clearly influence the distribution of phosphate: (*a*) the much greater protein concentration (and therefore smaller quantity of available water) in the corpuscles than in the plasma, (*b*) the Donnan equilibrium[1] between the cells and plasma. Thus, (*a*) plasma contains about 91·5 cc. of water per 100 cc., corpuscles some 65 cc., so that a substance present in simple aqueous solution would be distributed between 100 cc. plasma and 100 cc. of corpuscles in the ratio 140 : 100; (*b*) taking the Donnan ratio, r, for the distribution of univalent anions between the water of plasma and corpuscles of normal venous blood at p_H 7·4, to be 0·76 [Henderson, Bock, Field and Stoddard, 1924] and remembering that at this p_H phosphate ions are about four-fifths HPO''_4 and one-fifth $H_2PO'_4$, calculation indicates a theoretical ratio of 165 : 100 for the distribution of inorganic phosphorus

[1] The red cell appears to be freely permeable to the *inorganic* phosphate of the blood.

between 100 cc. water of plasma and 100 cc. water of corpuscles. This value, however, does not take into consideration the large difference in the amount of water in the two phases (see (*a*)). If we now correct for this we find

$$\frac{\text{concentration of inorganic P per 100 cc. plasma}}{\text{concentration of inorganic P per 100 cc. cells}} = 2{\cdot}32 : 1.$$

The average of the results obtained with foetal blood (plasma concentration : corpuscle concentration = 2·44 : 1) is in much closer agreement with this theoretical value of 2·32 : 1 than is the mean of the experimental findings for the blood of adults, given in Table I, in samples centrifuged in absence of anticoagulant (plasma concentration : corpuscle concentration = 1·71 : 1) or in presence of heparin (plasma concentration : corpuscle concentration = 1·67 : 1). In fact the latter values are not far removed from the theoretical value of 1·85 : 1 which is obtained if the assumption is made that the inorganic phosphate ions in blood are all univalent. There is, however, no reason to believe that this is the case. Does then the mere act of removing blood from a vein bring about (possibly as a result of changes occurring in the first stages of clotting or its prevention) a breakdown of the true *in vivo* equilibrium to a factitious equilibrium with a considerably lower plasma value than that which exists in the blood when it is circulating? The figures shown in Tables I and II support this hypothesis to some extent, though we cannot lay too much stress on a comparison between foetal blood and adult blood taken directly from active circulation. Foetal blood has inevitably been stagnant for some minutes in the vein, and its Donnan ratio, r, has not been directly determined and may be slightly different from that of adult venous blood.

Effect of temperature on r. Marrack and Thacker [1926, 2] found that when plasma was separated from whole blood at 38° or at 20° and its p_H then determined at 38°, there was, in normal subjects, an average increase of only 0·02 in the p_H of the plasma separated at 20°. Martin and Lepper [1926] found that the effect of temperature (with the concentration of total CO_2 constant) was the same upon the c_H of corpuscles and corresponding plasma. It would therefore appear that there is little interchange of ions between the corpuscles and the plasma in cooling from 38° to 20°, and thus the Donnan ratio r is probably not markedly affected by cooling the blood over this range.

Apart from the use of umbilical cords, we are not acquainted with any practicable means, applicable to the human subject, for obtaining plasma direct from the vein and thus being able to determine the true *in vivo* concentration of inorganic phosphate in the plasma. For working purposes we have taken the inorganic phosphate concentration in the plasma of venous blood centrifuged *in vitro* as a measure of the true concentration of inorganic phosphate in the plasma of blood entering the kidney, although in reality the latter value may be as much as 20 % higher than the former.

State of inorganic phosphorus in the plasma.

Is the inorganic phosphate of blood present in a diffusible form, or is it, like calcium, present partly in the form of ions, and partly in the form of an unionised, indiffusible compound?

Holt, La Mer and Chown [1925] brought forward evidence to show that normal blood-serum was apparently supersaturated with tertiary calcium phosphate to the extent of more than 200 %. They found, however, that artificial solutions containing $Ca_3(PO_4)_2$ remained supersaturated for days, as was also shown by Marrack and Thacker [1926, 1] for artificially supersaturated solutions of $CaCO_3$. A possible explanation of this apparent supersaturation is that the calcium phosphate is present in serum or plasma in a colloidal form, and, indeed, Eichholtz and Starling [1925], from work on the excretion of phosphate by the heart-lung-kidney preparation, came to the conclusion that a great part of the inorganic phosphate present in normal serum is present as a non-ionised, colloidal calcium compound, and not in the form of sodium orthophosphate.

The balance of evidence up to the present is, nevertheless, against the view of these authors, at least in so far as mammalian blood is concerned. Marrack and Thacker [1926, 1] find no evidence of the presence of colloidal phosphate or carbonate in artificially supersaturated solutions, whilst Cushny [1919] and Pincus and his collaborators [1923, 1926] consider that the whole of the inorganic phosphorus of serum is present in the form of ions or ultrafilterable electrolytes.

We have carried out a number of ultrafiltrations of both plasma (heparinised or oxalated) and serum, and have found in all cases that a large proportion of the inorganic phosphate of the plasma is ultrafilterable through cellophane (which is, on the whole, a more resistant ultrafiltering medium than collodion and would be more likely to effect the complete retention of substances in the colloidal state). The ultrafilter used is that described by Aitken and Kay [1927][1], and a micro-modification of the Bell and Doisy or of the Briggs method was used for inorganic phosphorus. In all except 3 (out of 32 determinations) with rabbit's or human plasma or horse serum (either heparinised or oxalated) there was slightly less inorganic phosphorus in the ultrafiltrate

Table III. *Ultrafiltration experiments. P mg. per 100 cc.*

Kind of plasma	Ultra-filtration membrane	No. of exps.	Inorg. P in original plasma	Inorg. P in residual plasma	Inorg. P in ultra-filtrate	Percentage of ultra-filterable inorg. P*
Rabbit heparinised	Cellophane	8	3·8	—	3·1	75
Rabbit oxalated	,,	4	3·4	—	3·5	95
Human heparinised	,,	5	3·8	4·0	3·3	80
Human oxalated	,,	4	4·0	4·1	3·8	90
,,	Collodion	4	4·0	4·2	3·5	80

* To nearest 5 %. Corrected for protein concentration.

[1] For work with phosphate it is essential that this ultrafilter should be nickel plated It is advantageous to use a double thickness of cellophane.

than in either the original or the residual plasma. Usually about one-third of the volume of the plasma in the ultrafilter came through during the experimental period. Results are summarised in Table III.

Plasma was also dialysed at 0° in collodion sacs against from 1 to 3 volumes of distilled water or 0·9 % NaCl, and the inorganic phosphate concentration within and without the membrane determined after equilibrium had been reached. In no case was protein found in the dialysate.

Table IV. *Dialysis experiments.*

Fluid dialysed	Dialysed against	Time hours	P mg. per 100 cc. Inorg. P in sac	P mg. per 100 cc. Inorg. P in dialysate
Rabbit's heparinised plasma	0·9 % NaCl	18	1·4	1·3
" " "	"	60	2·1	1·8
" " "	"	18	1·2	1·25
" " "	Distilled water	18	0·7	1·0
Rabbit's oxalated plasma	0·9 % NaCl	18	1·1	1·4
" " "	Distilled water	18	0·8	0·7
Human " "	"	18	0·8	0·9
" " "	"	18	1·4*	1·7*
			1·3*	1·5*
		Mean	1·17	1·27

* Duplicate dialyses.

It is perhaps permissible to take a mean of these findings, when it will be seen that on the whole, allowing for the protein content within the sac, the inorganic phosphate of normal human or rabbit plasma may be considered to be fairly completely dialysable.

Since this work was completed a paper by Grollman [1927] has appeared in which he states that the inorganic phosphate in the serum of blood from normal dogs or pigs is completely ultrafilterable. He has found, however, that the addition of $CaCl_2$ to plasma, or the hypercalcaemia produced by parathyroid injections renders some of the inorganic phosphorus no longer filterable.

Many of our phosphate determinations have been done by Briggs's method, after precipitating the proteins with trichloroacetic acid. Briggs's method entails the use of fairly strong acid (about 0·5 *N*) and the question arises as to whether some or all of the apparent "inorganic" phosphate of blood may not, in fact, result from the breakdown of an organic phosphorus compound unstable in acid solution, such as phosphagen [Eggleton and Eggleton, 1927], a compound of creatine and phosphoric acid.

Using precautions to avoid contact with acids for more than very short periods and that at low temperatures, we have been unable to detect any evidence of such a substance being present in blood, and Eggleton [1927] states that phosphagen is in all probability absent from blood.

The foregoing experiments have of necessity been carried out upon oxalated or heparinised samples of blood or plasma *in vitro*. It may be objected therefore that the results do not truly indicate the state of inorganic phosphate in

the normal circulating blood. Nevertheless, until definite evidence in favour of this objection is produced, it seems to us justifiable to accept the data furnished by the above experiments as showing that the "inorganic" phosphate of normal plasma is present mainly as a simple, ultrafilterable and dialysable salt of orthophosphoric acid.

The presence and quantity of phosphoric esters in the plasma.

Feigl [1917] showed that, in addition to the inorganic phosphate of the serum, organic acid-soluble phosphorus compounds, of unknown constitution, were also present in relatively small amount. This has been confirmed by other workers. Since, however, there is a large amount of organic phosphorus in the corpuscles, it is possible that the finding of very small and varying amounts of it in the serum or plasma might simply be an indication of slight haemolysis[1] or of incomplete removal of the corpuscles on centrifuging. Martland and Robison [1926] and Kay [1926, 1], avoiding these errors as far as possible, have shown that varying amounts of acid-soluble phosphoric esters are present in plasma freed from formed elements, and in which there was no sensible haemolysis, and that these esters are hydrolysable by body phosphatases. In particular it has been shown that the plasma-esters are probably completely hydrolysed by an extract of kidney.

Using normal human plasma, or the plasma of nephritic patients, obtained by centrifuging the blood at 7000 revs. per min. for 15 minutes, and free from visible haemolysis, we have obtained results completely confirmatory of the findings of the authors just quoted. These are summarised in Table V.

Table V. *Concentration of ester-P in plasma and urine.*

		Plasma organic P mg. per 100 cc.	Urinary organic P	
			mg. P per 100 cc.	mg. P per hour
Normals (17)	highest	0·9	2·8	3·0
	lowest	0·0	0·0	0·0
	mean*	0·33	0·8	1·2
Nephritics (31)	highest	2·2	2·1	3·4
	lowest	0·0	0·0	0·0
	mean*	0·52	0·6	0·7

* The majority of the determinations are grouped around the mean.

In normal human plasma we find an average of 0·33 mg. phosphorus as phosphoric esters in 100 cc., in nephritic plasma there is slightly more. Patients with nephritis appear to show some retention of organic phosphorus, as well as of inorganic phosphate, nitrogen, etc., in the plasma. There also appears to be some difficulty in the excretion of organic phosphorus by the

[1] Mâcheboeuf [1927] claims to have isolated phosphoglyceric acid from serum. This substance is known to be present in red cells, and since Mâcheboeuf used the serum from defibrinated blood, in which there is notoriously a good deal of haemolysis, his results cannot be completely accepted until they are repeated on serum or plasma obtained with more adequate precautions.

nephritic kidney, despite the greater "pressure-head" in the plasma. (All the urine figures in Table V are for urine collected in the period 8.30–10.30 a.m. after a very light breakfast at 6 a.m.)

We have, then, considerable experimental foundation for the conclusion that in normal plasma the phosphorus is present in two forms: (*a*) the major portion, inorganic, mainly if not completely dialysable, (*b*) a relatively small amount of organically combined phosphorus, probably about 10 % of the total acid-soluble phosphorus of the plasma.

The importance of this definite information with regard to the presence and approximate quantity of ester phosphorus in the plasma will be evident when the hypothesis of Eichholtz, Robison and Brull is under consideration.

Uptake of phosphorus from the intestine.

In the food phosphorus occurs entirely as the salts or esters of phosphoric acid. In no case, as far as we are aware, has phosphorus been demonstrated to occur naturally, either in plant or animal tissues, in any other state of oxidation[1].

There is no evidence at present that organic phosphorus compounds (with the possible exception of lipins) are taken up as such into the blood through the wall of the intestine. Hydrolysis, at least in the adult, appears to occur invariably, the powerful phosphatase in the succus entericus and in the intestinal mucosa being the active agent.

We have made several attempts to raise the organic phosphorus content of the plasma by the ingestion of one of the most stable esters of phosphoric acid. Fairly large doses of sodium glycerophosphate have been taken by mouth by several individuals, without any detectable increase in the organic phosphorus of the plasma or the whole blood, though increases in the inorganic phosphate followed invariably.

The protocols of a typical experiment are given below: 8 a.m., 300 cc. water taken; 8.30 a.m., light breakfast; 9.0 a.m., first urine sample; 25 g. sodium

Table VI. *Effect of ingestion of glycerophosphate on plasma and urinary phosphates.*

	Plasma mg. P per 100 cc.			Urine	
Time of taking blood	Inorg. P	Organic P	Time of collection	Inorg. P mg. per hr.	Mean plasma-P
10.15 a.m.	2·8	0·34	9.0 –10.30	16·3	2·8
11.20	3·6	0·31	10.30–11.30	13·5	3·2
12.20 p.m.	4·40	0·31	11.30–12.30	59·5	4·1
1.20	4·36	0·28	12.30– 1.30	69·1	4·38
2.20	4·30	0·34	1.30– 2.30	70·2	4·33
3.20	4·26	0·40	2.30– 3.30	70·2	4·28
4.20	4·23	0·32	3.30– 4.30	80·3	4·24
5.20	3·98	0·25	4.30– 5.30	75·6	4·1

[1] The statement occasionally seen in text-books that the phosphates of the urine are derived in part by the oxidation of the phosphorus of the phosphoproteins is fallacious, since the phosphorus in these proteins is already fully oxidised, and requires hydrolysis only to become free phosphate.

glycerophosphate in 500 cc. water. From this hour till 5.30 p.m., samples of blood and urine were taken and 250 cc. water was drunk hourly. Results are shown in Table VI.

Altogether nine similar experiments were performed, in some of which additional determinations were made on the total acid-soluble phosphorus in whole blood, and on the lipin phosphorus. The results seem to show quite definitely that when the relatively stable phosphoric ester, sodium glycerophosphate, is present in the gut (*a*) there is no demonstrable rise in the organic phosphorus of plasma or of whole blood, (*b*) there is a definite rise in the inorganic phosphate of the plasma followed by a rise in the rate of urinary excretion of inorganic phosphate. Moreover, a physiological increase in the inorganic phosphate of the plasma does not affect the level of the plasma phosphoric esters, though the rate of urinary excretion may rise fivefold. There is therefore, at least under these experimental conditions, no correlation whatever between the amount of phosphoric esters in the plasma and the rate of inorganic phosphate excretion by the kidney.

Phosphate excretion by the kidney.

We now proceed to examine the suggestion of Eichholtz, Robison and Brull mentioned earlier in this paper that the inorganic phosphorus of normal urine has its immediate origin in the acid-soluble organic phosphorus compounds of the plasma, by hydrolysis of these in the kidney, a hypothesis which at the same time provides a logical primary function for the very active phosphatase known to be present in kidney tissue. Briefly stated, the experimental basis for this suggestion is as follows: these authors added sodium glycerophosphate to the fluid perfusing the heart-lung-kidney preparation, and found that whereas the kidney was previously unable to concentrate inorganic phosphorus, it now began (after an interval) to excrete both organic and inorganic phosphorus at definitely higher concentrations than those in the circulating (defibrinated) blood.

On examining this hypothesis in the light of these and other experimental findings the following conclusions may be drawn: (1) there is quantitatively more than enough phosphatase present in the human kidney to bring about the necessary hydrolysis [Kay, 1926, 2]; (2) putting the organic phosphorus of the plasma at 0·3 mg. P per 100 cc. (see later section) there is sufficient substrate present to provide, on hydrolysis, the whole of the inorganic phosphate of the urine; (3) there is no doubt that phosphoric esters, added to the perfusing blood, are to a marked extent hydrolysed in the heart-lung-kidney preparation. The above mentioned authors, however, make one or two assumptions that should not pass without comment. They assume that the kidney is the only tissue in their preparation which is capable of hydrolysing phosphoric esters. This is not the case, since blood itself, heart muscle to a slight degree, and lung tissue to a much larger degree also possess this hydrolytic power. Nevertheless, it is probably true that in their preparation the

kidney is quantitatively the most important tissue as far as phosphatase action is concerned. They also assume that a relatively small rise in the inorganic phosphate of the blood would hardly affect the rate of excretion of phosphate by the kidney. The findings of many other workers, and even their own figures[1] bear witness that this is a very doubtful assumption. If all the serum inorganic phosphate figures given by these authors are entered on their charts, it will be seen that there is a closer correlation between the level of inorganic phosphorus in the serum and the rate of excretion of inorganic phosphorus in the urine, than between the latter and the level of organic phosphorus in the serum. Our interpretation of the results of Eichholtz, Robison and Brull is that the phosphatases in the various tissues—the chief hydrolysing tissue being, in their experiments, the kidney—have slowly decomposed the circulating glycerophosphate, thereby raising the inorganic phosphate concentration in the serum above a threshold level, and, by continuous hydrolysis, have kept it at a high but slowly sinking level as long as any glycerophosphate has been available. In glucose tolerance tests it is frequently found that once the kidney threshold is overstepped, glucose continues to be excreted at plasma values which are normally below the threshold, and this seems to have occurred in one or two of these authors' experiments with phosphate. If the urinary inorganic phosphate is derived from the plasma organic phosphate it would be expected that the higher the plasma organic phosphate, the greater the rate of phosphate excretion. This relation is not seen either in their results, or in our own with the intact animal, in which after the injection of organic phosphorus we have repeatedly found that the excretion of inorganic phosphate may be greater with a low value for plasma organic phosphorus than with a high one.

We are definitely of opinion that in the normal human subject or in the normal rabbit the source of inorganic phosphate in the urine is not the organic but the inorganic phosphorus of the plasma, and that kidney phosphatase is not primarily concerned in the appearance of urinary phosphates. We will first summarise our reasons for these conclusions and then give the experimental evidence on which they are based.

Our reasons are as follows.

(1) Although the inorganic phosphate of the urine may be markedly increased by increasing the intake of either inorganic or of organic phosphate by the mouth, it does not appear possible to raise the organic phosphorus of the plasma by this means; *i.e.* the rate of inorganic phosphorus excretion may be increased whilst the level of organic phosphorus in the plasma remains normal.

[1] Thus, in their Table IV, after the injection of 50 mg. P as sodium phosphate the excretion of inorganic phosphate rises from 2 units to 30 units in 10 minutes, with a rise of inorganic P in the serum from 4·0 to 7·8 mg. P per 100 cc. With the serum inorganic P still at a high level, 50 mg. *organic* phosphorus causes a rise in phosphate excretion in the urine from 17·9 to 32 units in 10 minutes, but the maximum rate of excretion of 42 units takes 1 hour to attain, and is reached with the organic phosphorus in the serum actually falling.

(2) On increasing the organic phosphorus content of the plasma of an intact animal by intravenous injection of a phosphoric ester readily hydrolysable by kidney phosphatase (and, therefore, by other body phosphatases), the kidney excretes organic phosphate in a concentrated form. At the same time the excretion of inorganic phosphate increases, but only *pari passu* with the increase in inorganic phosphate in the plasma.

(3) Conditions have frequently been observed in which, while the inorganic phosphate in the urine was increasing, the organic phosphate in the plasma was definitely decreasing. In all these cases the rate of excretion of organic phosphate by the kidney was related to the level of organic phosphorus in the plasma, while the rate of excretion of inorganic phosphate clearly depended on the level of inorganic phosphate in the plasma.

(4) Organic phosphate injected into the circulation of a nephrectomised animal is hydrolysed at about the same rate as if it had been introduced into the circulation of an intact animal; *i.e.* hydrolysis of phosphoric esters in the circulating plasma goes on quite well without the intervention of the phosphatase of the kidney at all.

(5) There is an approximately linear relationship between the inorganic phosphate level in plasma and the rate of its excretion in the urine, whether the amount of organic phosphorus in the plasma is normal or is artificially increased.

The experiments on which these conclusions rest will now be briefly described.

A. Large male rabbits were given intravenous injections of (*a*) inorganic phosphate at p_H 7·4, (*b*) neutral sodium glycerophosphate, (*c*) neutral sodium hexosephosphate. Samples of blood were taken, in each case, before, and at intervals after the injection, and specimens of urine were obtained by catheterisation (bladder washed out each time with warm water in order to secure the whole of the urine secreted).

(*a*) The results of a typical experiment with inorganic phosphate are shown in Fig. 3.

Doubling the plasma concentration of inorganic phosphate results in an increase of rate of excretion of about tenfold. This immediate response of the kidney to a rise in plasma content supports very strongly a direct relationship between the plasma and the urinary values. In any case no appreciable amount of organic phosphorus was present in the plasma either at the beginning or during the course of these experiments.

(*b*), (*c*). Sodium glycerophosphate and fermentation sodium hexosediphosphate behave very similarly. The rapid rise in the plasma level of organic phosphorus after injection is followed by a fairly rapid fall. At the same time the inorganic phosphate in the plasma increases, undoubtedly as the result of hydrolysis of the injected ester by the tissue phosphatases. If the inorganic phosphate of the urine results from the hydrolysis of the organic phosphate of the plasma by kidney phosphatase, there should be a maximum urinary

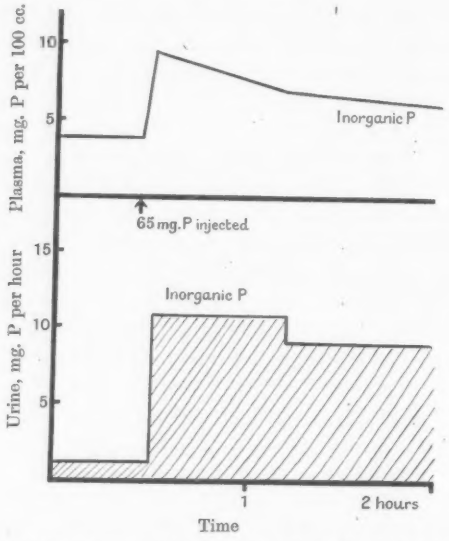

Fig. 3. Rabbit. Intravenous injection of sodium phosphate (at p_H 7·4).

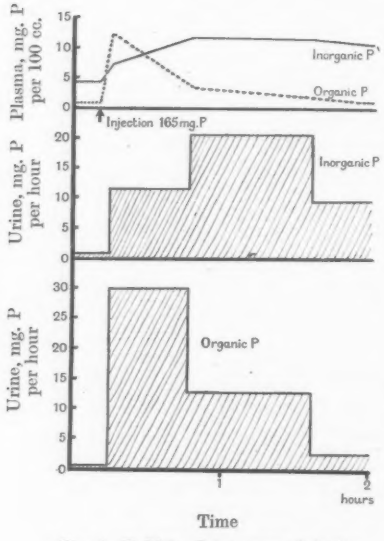

Fig. 4. Rabbit. Intravenous injection of sodium glycerophosphate.

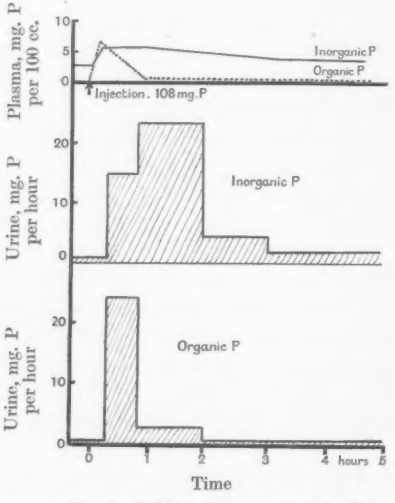

Fig. 5. Rabbit. Intravenous injection of sodium hexosediphosphate (10 mg. inorganic P 98 mg. organic P).

excretion of inorganic phosphate during the first hour, when the organic phosphate of the plasma is at its maximum. Actually the excretion of inorganic phosphate by the kidney in the first hour is nearly doubled in the second hour (Figs. 4 and 5) although the concentration of organic phosphorus in the plasma is then approaching its pre-injection level. It will be noted that organic phosphate appears in the urine in considerable amounts when its concentration in the plasma is increased. In its excretion it appears to behave as a substance with very low threshold.

B. Normal men drank a solution of 25 g. of sodium glycerophosphate in 400 cc. water. Samples of blood and urine were collected before, and at 2-hourly intervals afterwards. A moderate water intake was allowed, but no attempt was made to produce a diuresis. A typical experiment has been given in Table VI. In none of nine experiments was there any appreciable

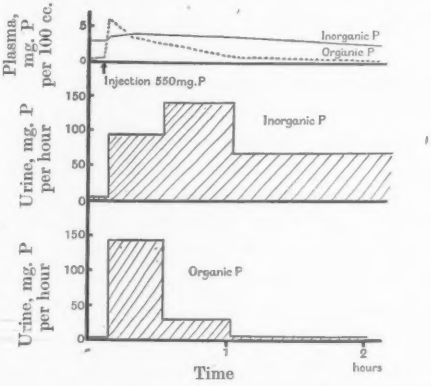

Fig. 6. Normal man. Intravenous injection of sodium glycerophosphate.

rise in the plasma content of organic phosphorus. It may be concluded that ingested sodium glycerophosphate is completely hydrolysed during absorption. It is virtually equal, in its effect on blood and urinary phosphates, to an equivalent amount of ingested inorganic phosphate, the rates of change of concentration of plasma and urinary phosphates after ingestion of sodium glycerophosphate agreeing fairly well with those reported by Wigglesworth and Woodrow [1923] to follow the ingestion of sodium phosphate.

C. Since it was not possible to raise the level of plasma organic phosphorus by ingestion, normal men were given sodium glycerophosphate intravenously, the plasma inorganic and organic phosphate and the urinary excretion being followed. The findings with rabbits described in A above have been abundantly confirmed for normal human adults. A typical experiment (one of ten) is shown in Fig. 6[1].

[1] In two experiments the lipin-P in the blood was also followed. In neither case was there a rise in the amount present.

In these experiments, as before, it was repeatedly observed that the rate of urinary excretion of inorganic phosphate could never be correlated with the concentration of the organic phosphate, but always with that of the inorganic phosphate in the plasma.

In view of our experimental findings, a short comment on two other theories of the origin of urinary phosphates may be made here. The first is that of György [1925] who believes that the urinary phosphates have their origin, for the major part, in the organic phosphoric esters of the kidney tissue. This author has studied the autolysis of kidney tissue *in vitro*, and finds that the free phosphate increases, but gives no evidence, other than this, for his views. We think it is just as legitimate to conclude that the carbon dioxide of the expired air is derived from the combustion of the carbon compounds of lung tissue. While it is possible, and indeed probable, that a small portion of the urinary phosphate is derived from the breakdown of kidney tissue, it seems to us in the highest degree unlikely that upwards of a kilogram of kidney tissue is autolysed in the body per day to provide the 1·2 g. of urinary phosphorus excreted in man: this result follows if György's theory is worked out arithmetically.

The second theory, which belongs to a different category of probability, is that of Jost [1927]. In an able investigation of the biological significance of phosphoglyceric acid in the red corpuscle, this author comes to the cautious conclusion that if any part of the urinary phosphate is derived from the blood phosphoric esters, this compound is its most important mother substance. We agree. In normal blood, however, it appears to be entirely within the corpuscle, and nothing we have tried, short of laking the cells, is able to bring it, in the unhydrolysed form, into the plasma. Until, therefore, we have some definite evidence that it can emerge, under physiological conditions (apart of course from the normal breakdown of red cells which is continually proceeding), from the cell and become hydrolysed by the kidney enzyme we find it difficult to accept the suggestion that it is an important source of the urinary phosphate.

Is there a renal threshold for phosphate?

In the experiments described under B above, the inorganic phosphate level in plasma and the corresponding rate of urinary excretion of inorganic phosphate have been determined for six normal individuals (9 experiments, 23 values). If the plasma values are plotted against the rate of excretion (Fig. 7), the points are found to lie fairly close to a straight line which, if produced, cuts the abscissa at a plasma phosphate value of 2·8 mg. P per 100 cc.

If the results obtained following the intravenous injection of sodium glycerophosphate in man are similarly plotted, the line cuts the axis at a plasma value of about 3·5 (Fig. 8). The more scattered appearance of the points in this group of experiments is due, at least in part, to the more rapid changes which are occurring in the level of inorganic phosphate in the plasma,

which render the mean plasma value during any given period of urinary excretion a somewhat uncertain figure[1]. As, however, the 34 points recorded in Fig. 8 are from as many as 9 persons of different ages and sex, the concordance is satisfactory. It may be concluded that when the inorganic phosphate content of the plasma rises above a certain level, the excretion of phosphate by the kidney either begins, or changes fairly suddenly from a very low rate to a much higher one. We use the word "threshold" to describe the level of a urinary constituent in the blood at which this *sudden increase* in the excretory rate takes place. Above this level, as is demonstrated by Fig. 7, the kidney excretes phosphate at a rate approximately proportional

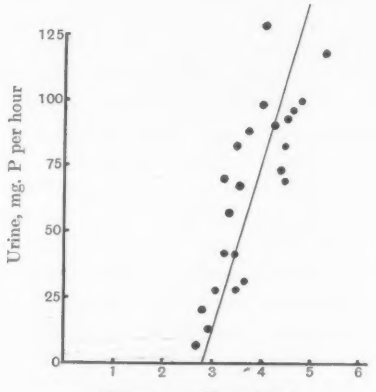

Fig. 7. Rate of excretion of inorganic phosphate and plasma concentration, after ingestion of sodium glycerophosphate. (Six normal adults.)

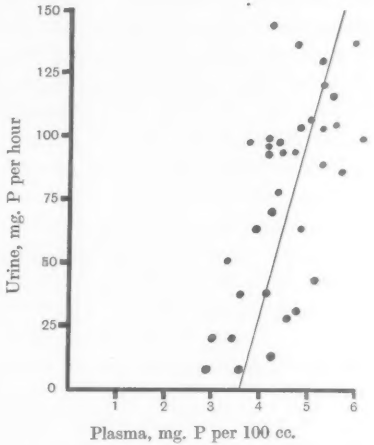

Fig. 8. Rate of excretion of inorganic phosphate and plasma concentration, after intravenous injection of sodium glycerophosphate. (Nine subjects.)

to the extent of the increase of plasma phosphate above the threshold level. The graph in Fig. 7 is represented by the equation $U = r\,(P - a)$ where U is the rate of excretion of phosphorus in mg. per hour, P is the concentration of inorganic phosphorus in the plasma in mg. per 100 cc., a is the threshold in the same units, and r is the excretory ratio—a constant. In both Figs. 7 and 8 the slope of the line is the same, thus r is the same in both and works

[1] Also the subjects varied in weight, and therefore presumably in kidney size and excretory capacity, so that the excretion per hour uncorrected by some function of the weight is a somewhat unsatisfactory ordinate. Nevertheless, we consider that there are fewer objections to its use than to the use of the "excretion per *kilogram* per hour" as the ordinate. The metabolism in any case is not a linear function of the body weight, nor is the weight more than a very dubious estimate of the amount of kidney tissue.

out at about 60. This is approximately the value found for r for urea excretion in man, under similar conditions, by Hewlett, Gilbert and Wackett [1916] (see Adolph [1925]).

Wigglesworth and Woodrow [1923] ingested inorganic (sodium) phosphate and investigated the subsequent changes in the blood phosphate levels and rates of urinary excretion. We have recalculated their data by applying the formula for converting blood phosphate figures to plasma values. Their threshold ("a") value is about 3·4 mg., and their r works out at about 50.

The results of these workers, and our own, suggest a fairly high threshold for inorganic phosphate, to wit, one only slightly below the normal plasma level. A threshold, in the stricter sense of the word, implies that below that level inorganic phosphate is not excreted. Addis, Meyers and Bayer [1925] stated that there was no reference in the literature to the complete absence of phosphate from the urine, which with a threshold of the above value one might expect to have been observed. But under normal and under the majority of abnormal conditions, the inorganic phosphate never goes below the threshold value. This occurs however in insulin hypoglycaemia, in overbreathing and occasionally in rickets. Sokhey and Allen [1924] showed that in both normal and diabetic dogs insulin hypoglycaemia was accompanied by a greatly diminished rate of excretion of phosphate in the urine, traces only being found for some hours after insulin administration. It is well known that insulin produces a definite, though not a very severe, hypophosphataemia, so that it would appear that the phosphate threshold in dogs is also not very far below the normal plasma level. In man, Haldane, Wigglesworth and Woodrow [1924] found that after a period of overbreathing the inorganic phosphate of the blood fell from the normal value of 3 to 0·9 mg. P per 100 cc. or even lower. During one experiment, whilst the blood inorganic phosphate fell from 3·2 to 0·95 mg., the rate of excretion in the urine fell from over 20 mg. per hour to about 2 mg. With shorter periods for urinary collection, even lower excretory values might have been attained. It is likely, however, that even with a very low blood phosphate, *i.e.* one far below the threshold level, small quantities of inorganic phosphate, produced during the functional wear and tear of the phosphorus-rich kidney tissue itself, will continue to appear in the urine. This may account for the fact that urine completely free from phosphate has not yet been reported.

If a phosphate threshold actually exists, as appears probable, its level even for the same individual will differ at different times. Havard and Reay [1925] have found that the inorganic phosphate of the blood shows a distinct seasonal variation. A change in the kidney threshold for phosphate must clearly accompany, or possibly be the cause of, this variation, otherwise there would be a very serious loss of phosphate from the body in June, July and August when the blood inorganic phosphate was at its seasonal maximum of from 3·6 to 4·0 mg. P per 100 cc. blood (= plasma 4·0 to 4·5 mg. per 100 cc., cf. Fig. 2).

The fact that a large proportion of the phosphate of the plasma is dialysable and ultrafilterable seems to dispose of the possibility that the threshold is due to the presence of most of the "inorganic" phosphate of plasma in a form which, owing to its colloidal nature, is held back at the glomeruli.

Rate of disappearance of injected glycerophosphate from the circulation.

In a number of cases blood was taken just before, and at intervals after, the intravenous injection of sodium glycerophosphate in normals and in nephritics. The rise in organic phosphorus took place entirely in the plasma, in no case was there a measurable increase in the organic phosphorus of the corpuscles. There was a rapid disappearance of injected glycerophosphate from the blood; usually within 5 minutes more than three-quarters of the injected salt was no longer circulating. In this time it had not been excreted in the urine, nor had it been hydrolysed into inorganic phosphate in the blood. It must therefore have been taken up by the tissues [see Underhill, 1923].

Typical figures illustrating this point are given in Table VII.

Table VII. *Rate of disappearance of injected glycerophosphate from the plasma.*

No. of exp.	Type of case	Time after injection (mins.)	mg. P per 100 cc. Rise in organic P of plasma	mg. P per 100 cc. Theoretical* rise in plasma	Percentage disappeared
40	Normal man	8	5·0	23	80
42	,,	5	4·7	26	80
45	,,	5	5·4	22	75
41	Nephritic	6	3·6	17	80
80	,,	3	5·0	19	75
81	,,	5	4·9	19	75
38	,,	5	4·5	19	75
	,,	10	3·1	19	85
	,,	60	1·2	19	95
58	Normal rabbit	3	47	170	70
	,,	40	19	170	90
	,,	75	12	170	95

* Blood volume taken as 1/20th body weight.

Phosphate excretion and urinary volume.

Cushny [1926] states that in diuresis the rate of excretion of urinary phosphate rises only slightly, if at all. We have found that, following the same stimulus, the excretion of both organic and inorganic phosphate in the urine of normal individuals is independent of the amount of water excreted. In Table VIII is found a series of consecutive observations on ten normal individuals.

The independence of inorganic phosphate excretion on urinary volume is also strikingly shown in certain results recorded by Havard and Reay [1926] in which the diuresis reached 1200 cc. per hour, and the actual concentration of inorganic phosphate in 100 cc. urine was less than that in blood.

Table VIII.

Inorganic P excreted in 1 hour following intravenous injection of 5 g. sodium glycerophosphate in a volume of from 100 cc. to 10 cc. of aqueous solution.

Exp. No.	Urine volume in cc.	Inorganic P excreted mg.	Organic P excreted mg.
25	500	105	60
26	229	129	47
28	212	118	93
31	43	118	78
33	70	98	62
35	93	110	70
40	600	107	83
42	221	93	88
45	246	115	137
95	199	109	86
	Mean	110	80

Relation between plasma concentration and rate of urinary excretion.

In our discussion of the possible threshold for phosphate excretion, and indeed throughout most of the present paper, we have assumed that the plasma and not the whole blood concentration of a urinary constituent gives a truer measure of the pressure head with which the kidney has to deal. When, however, the details of transference of a urinary constituent out of the blood to or through the kidney cells are considered, the real value of this measure becomes somewhat doubtful. We mention two only of the questions that arise.

(1) Do the conditions of changing concentration in the blood circulating through the capillaries supplying the excretory elements permit of equilibration of diffusible substances between plasma and corpuscles? Even assuming that there is no such movement across the corpuscle boundary, the pressure head of the effective concentration of any one substance in the plasma must, owing to changes in the protein concentration of the latter, be continually altering in a complicated way during the progress of the blood through the renal vessels. We may know the initial plasma concentration of any given urinary constituent in the renal artery (approximately equal, for most substances, to that in venous plasma), but we do not know its final concentration in the renal vein, which may or may not be considerably lower than that in the artery. Even if we did know the final concentration, owing to such changes the mean value could not be taken with certainty as representing the effective plasma concentration. To be taken into account also is the probable continuous back-diffusion of certain of the urinary constituents from the concentrated urine in the tubules back again into the plasma, owing to partial permeability of the tubule walls [Rehberg, 1926]. This back-diffusion would be expected to depend on the rate of excretion of water, so that urinary volume may enter as another factor into our conception of the threshold level.

(2) Where a corpuscle is squeezing through a narrow capillary, may there not be direct interchange of substances between corpuscular contents and the cells of the capillary wall, particularly if the capillary is part of an excretory

or secretory system? Krogh [1922] has shown that in certain cases there is considerable mechanical squeezing of the corpuscles in passing through the capillaries. It is just conceivable that the phosphoric esters within the red cells may in certain conditions leave the cells to appear in the urine either as unhydrolysed ester or as inorganic phosphate.

The following conclusions with regard to the excretion of phosphate are, we believe, in agreement with the experimental facts found by ourselves and by others. The inorganic phosphate in normal urine arises from two immediate sources: (*a*) a small, basal quantity excreted at a fairly constant rate no matter what the level of inorganic or organic phosphate in the plasma may be, produced during the normal wear and tear of the renal cells, being in a real sense endogenous and derived from the inorganic and organic acid-soluble phosphorus compounds of kidney tissue; (*b*) the main quantity, under normal conditions much larger than (*a*), derived immediately from the inorganic phosphate of the plasma, and appearing so long as the level in the plasma is maintained above a threshold value. This fraction (*b*) is actively excreted by the kidney. The amount excreted increases in linear proportion to the rise in the plasma level of inorganic phosphate above the threshold value, and has nothing to do directly with the plasma level of acid-soluble phosphoric esters. If by some means this latter value is artificially raised, the excess of ester is partly excreted unchanged, and partly slowly hydrolysed by all the active tissues of the body (including the kidney itself) and the resulting inorganic phosphate produced is excreted by the kidney in the usual way. The possibility is not denied that some of the ester circulating under abnormal conditions in the blood may, by being hydrolysed in the kidney, provide a small portion of the extra excretion of inorganic phosphate. Whereas, however, in the heart-lung-kidney preparation, in which the kidney is a relatively large fraction of the phosphatase-containing tissue, the quantity of inorganic phosphate so produced may be an appreciable fraction of the total inorganic phosphate excretion, these results cannot be applied to the intact animal where quantitative relationships of an entirely different order have to be taken into account.

SUMMARY.

1. Acid-soluble phosphorus in the plasma is present in two forms: (1) as a simple salt of orthophosphoric acid, of which at least 80 % is ultrafilterable and dialysable and therefore neither colloidal nor bound to the plasma colloids, (2) as organically combined (ester) phosphoric acid. The amount of (2) is usually about 10 % of the amount of (1) both in normal plasma and in that from patients with nephritis.

2. The amount of inorganic phosphate in the plasma, as determined by the usual methods, is less than the amount to be expected from the Donnan equilibrium between corpuscles and plasma.

3. The very low concentration of ester phosphorus in the plasma cannot be raised by taking phosphoric esters by mouth. It can be raised temporarily by intravenous injection of phosphoric esters.

4. In the intact rabbit or in man, there is no evidence in favour of the view that the ester phosphorus of the plasma controls the rate of excretion of inorganic phosphate. On the contrary, the evidence is all in favour of the control of such excretion by the plasma level of inorganic phosphate. There appears to be a renal threshold for phosphate excretion.

5. Within wide limits there is no relationship between the amount of urinary phosphate and the urinary volume.

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LXXXI. THE REDUCING POWER OF CYSTEINE.

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The physiologic significance of glutathione has been of the greatest interest ever since this compound was isolated by Hopkins [1921]. It has been shown to influence oxidative processes *in vitro*, and Dixon and Quastel [1923] and Dixon [1927] have published investigations concerning the oxidation-reduction potentials of glutathione and cysteine.

Kendall and Nord [1926] described some experiments concerning the reducing power of glutathione and cysteine and stated that both the —SH and —SS forms of these compounds are relatively stable. Cysteine and GSH cannot reduce indigo carmine and the —SS groupings cannot act as oxidising agents in a phosphate buffer of p_H 7·4. In the presence of indigo carmine, however, it was shown that the addition of hydrogen peroxide or sodium disulphide produces a third component in the solution which activates cysteine and GSH so that they can readily reduce indigo carmine and the —SS grouping can act as an oxidising agent; furthermore, the oxidation-reduction potential of the system is determined by the ratio of —SH to —SS if this third component is present in the solution. It was suggested that the third component is an oxygen or sulphur addition product of the —SH grouping.

Dixon and Tunnicliffe [1927] have recently criticised this work. They state that the published results do not warrant the conclusions and that the work was performed in an inaccurate manner with faulty technic and impure preparations. They characterised the suggested mechanism as novel and unnecessary for the explanation of the activity of the —SH and —SS groupings. Under the circumstances it appears necessary to reply with quantitative evidence to each of the many points raised by Dixon and Tunnicliffe.

Oxygen in the "oxygen-free" nitrogen.

Dixon and Tunnicliffe attribute the failure of Kendall and Nord to observe the reduction of indigo carmine by cysteine and GSH to the presence of oxygen in the nitrogen which was passed through the solutions. They estimate that the probable volume of gas passing through the solutions in 40 hours is 144 litres of nitrogen, and that the probable oxygen content of this is 15 cc., an amount sufficient to maintain the indigo carmine in the oxidised form.

They state that they have encountered difficulty in completely removing all traces of oxygen from nitrogen and assume that 0·01 % is a possible figure for the oxygen content of the nitrogen used by Kendall and Nord.

Before the investigation was started by Kendall and Nord an apparatus was constructed in such a manner that there was a large factor of safety in all details. For the purification of the nitrogen three Pyrex glass tubes, 2·5 cm. in diameter and 1 metre in length, were filled for a distance of approximately 60 cm. with fine copper-oxide wire. The section of the tube containing the copper was completely filled with the copper oxide so that there was no space between the oxide and the side of the Pyrex tube. The oxide was reduced with hydrogen; the three Pyrex tubes were placed side by side and each was wrapped with resistance wire and placed in a suitable asbestos-lined box so that a temperature between 300° and 400° could be constantly maintained. The diameter of the tubes was sufficient to permit a slow flow of gas through them and the subsequent reduction of copper oxide by passing hydrogen through the tubes in a direction opposite to that of the nitrogen showed that the copper was oxidised only in the first two tubes. Water was formed but rarely by reduction of copper oxide in the third tube. The ends of these Pyrex tubes were closed with a tapering glass seal to a 10 mm. glass tube bent downward making a **U**, and water forming in each of the copper-filled tubes would collect in this cooler **U**-tube giving an indication of the relative amount of copper oxide reduced by the hydrogen. Nitrogen, therefore, had to pass through approximately 180 cm. of metallic copper in a highly active form. The nitrogen passed through no rubber tubing in its course from the furnace to the solution. Copper tubing and glass with de Khotinsky's cement seals completely excluded all traces of oxygen.

Clark and Cohen [1923] have shown that this type of furnace is efficient for the removal of oxygen from nitrogen. We have many quantitative determinations proving the complete absence of oxygen from the nitrogen after passing through the furnace. A most severe criterion is the stability of 1 cc. of 0·004 *N* reduced indigo in 100 cc. of phosphate buffer, p_H 7·4. Such a solution will produce a reduction potential on the platinum electrode of approximately − 0·28 v. Oxygen-free nitrogen may be passed through such a solution constantly for 8 hours without a significant change in the potential and without the separation of any indigo. Similar experiments have been performed with other solutions exceedingly sensitive to traces of oxygen. A platinum electrode which has been treated with cathodic polarisation in a solution of cysteine is highly sensitive to traces of oxygen. The small amount of oxygen contained in 1 cc. of distilled water, added to this solution will cause a marked decrease in the potential; however, the oxygen-free nitrogen may be passed through this solution for many hours without any decrease in the potential (see Fig. 1, p. 654).

ACTIVATION OF THE —SH GROUP WITH HYDROGEN PEROXIDE AND IRON.

Dixon observed the reduction of indigo carmine with cysteine in 1922. In a recent paper [1927] Dixon also states that cysteine is capable of reducing indigo carmine. In order to show that oxygen, hydrogen peroxide, or sodium disulphide is unnecessary for the activation of the —SH group, Dixon and Tunnicliffe prepared an apparatus consisting of three compartments; in one was placed a neutral cysteine solution, in the second a small amount of indigo carmine and in the third a strong solution of alkaline sodium hydrosulphite. Air was removed from the apparatus with a water pump and nitrogen was admitted. This operation was repeated twice and the solutions then remained in a vacuum for 24 hours. The indigo carmine and cysteine were mixed and it was observed that the dye was reduced within 6 minutes. Dixon and Tunnicliffe concluded that this observation renders unnecessary the assumption of any oxygen addition product.

In order to test our samples of cysteine and indigo carmine a similar apparatus was prepared and amounts equivalent to those which Dixon and Tunnicliffe used were placed in the two arms. The apparatus was evacuated, filled with nitrogen and this operation was repeated twice. It was then allowed to stand in the vacuum for some hours. The solutions were mixed, but no reduction of the indigo carmine occurred during several hours. The original experiments of Kendall and Nord were then repeated. Indigo carmine was added to an oxygen-free solution of cysteine at a p_H of 7·4 and it was found that the solutions were not reduced in the electrode chamber within a period of 24 hours.

This result is diametrically opposed to that of Dixon and Tunnicliffe and in order to determine whether our sample of indigo carmine was unique we secured 15 samples of this compound from several retail chemical supply houses. They were prepared by the following manufacturing firms: four samples from Grüber, six samples from the National Aniline Company, three samples from Coleman & Bell, one sample from Merck & Company, one sample from Eimer & Amend (manufacturer not stated).

A 0·02 *N* solution of each of these samples was prepared and added to cysteine in electrode chambers. Not one of the samples was reduced by the cysteine. We may therefore conclude that the original sample of indigo carmine was not unique. We then prepared several other samples of cysteine, according to the method of Gortner and Hoffman [1925]. 2 kg. of human hair was hydrolysed with 4000 cc. of 20 % hydrochloric acid until the biuret reaction was negative, usually about 130 hours. The solution was filtered, sodium acetate was added and the cystine which separated was dissolved in hydrochloric acid, treated with norite, and reprecipitated with sodium acetate. The purification was continued until the crystals were colourless. The cystine was reduced by heating 24 g. for 20 hours with tin moss added to 75 cc. of concentrated hydrochloric acid and 100 cc. of water. The tin was removed

by hydrogen sulphide in a volume of 1800 cc. The solution was filtered through norite and was then made to a volume such that 1 cc. contained 12 mg. of cysteine. The solution, therefore, was 0·1 *N* in respect to cysteine and it generally was equivalent to a 0·35 *N* solution of hydrochloric acid. The hydrochloric acid was neutralised by an equivalent amount of oxygen-free *N* sodium hydroxide just previous to using the cysteine solution.

By a new method which has been developed in this laboratory by V. B. Dowler, it was shown that this solution contained no cystine when freshly prepared. After standing even in the hydrochloric acid solution for several weeks a small amount of cystine was present. The iron was not removed from this solution by the method of Sakuma although iron was removed from some of the samples of cysteine. With six samples of cysteine prepared as outlined it was impossible to reduce even 2 % of its molecular equivalent of indigo carmine.

Dixon and Tunnicliffe suggest that the main phenomenon observed by Kendall and Nord "is dependent on the presence of traces of iron present as impurities, perhaps as simple iron salts, perhaps as a cysteine-iron complex." It is difficult to interpret the suggestion that iron was the essential substance bringing about the reduction since no reduction was obtained in the absence of oxygen. However, the influence of iron on the reaction between cysteine and indigo carmine was investigated and it was found that when 1 cc. of 0·02 *N* ferrous sulphate or ferric chloride was added to a solution containing 6 cc. of 0·1 *N* cysteine and 3 cc. of 0·02 *N* indigo carmine in 100 cc. of phosphate buffer, p_H 7·4, there was no effect on the potential and the indigo carmine was not reduced.

That iron affects the reaction, however, may be readily shown and the experiment leaves little doubt concerning the reaction mechanism that is influenced by the iron. Indigo carmine, which is purified by recrystallisation from water, contains but minute traces of iron. Cysteine may be purified until it contains mere traces of iron, and when these two are mixed together no reduction of the indigo carmine occurs. If to such a solution hydrogen peroxide is added, or if air is admitted to the space above the solution, the indigo carmine is reduced but slowly. The cysteine and indigo carmine cannot be made to react with any appreciable velocity by the addition of hydrogen peroxide provided iron is removed as completely as possible. If traces of ferrous or ferric salts are added to a solution of cysteine in phosphate buffer, p_H 7·4, previous to the addition of hydrogen peroxide the indigo carmine is not reduced. If molecular oxygen or hydrogen peroxide is now added to the solution containing cysteine, indigo carmine and traces of iron, prompt reduction of the dye occurs. It is therefore evident that the function of iron is to decompose the hydrogen peroxide or activate molecular oxygen so that it can enter into the reaction between indigo carmine and cysteine.

This mechanism will be further discussed in connection with the influence of physical factors and the effect of iron expressed quantitatively is shown in Fig. 1 (p. 654). The sample of indigo carmine used by Kendall and Nord was

not free from iron; however, the presence of iron cannot explain the reduction of indigo carmine and the influence of iron in the reaction between oxygen and indigo carmine does not alter the conclusions concerning the necessity of activating the sulphydryl group. Dixon and Tunnicliffe conclude this discussion of the reduction of indigo carmine by the statement: "We consider that these results dispose of Kendall and Nord's contention that cysteine and glutathione cannot reduce indigo carmine in the absence of oxygen."

The results which have been outlined are completely at variance with those of Dixon and Tunnicliffe. However, the barrier so sharply separating the two conflicting results is surmountable. Each of the 15 samples of indigo carmine can be reduced by each of the six preparations of cysteine if traces of oxygen, hydrogen peroxide, sodium disulphide or other activating agent is added to the solution. It would therefore appear that if the objective is the preparation of solutions containing cysteine which are stable in the presence of indigo carmine Kendall and Nord have had the good fortune to attain that objective and Dixon and Tunnicliffe's failure must be regarded as a negative result. Until they have shown that at least one solution of indigo carmine free from oxygen is stable in the presence of cysteine, the conclusion is justified that their solutions are of such a nature that activation of the —SH group is brought about by an unrecognised impurity. As will be shown, they have not excluded oxygen as the probable activating agent, and we have found that sodium disulphide, colloidal sulphur, and some organic compounds, such as adrenalone, can form a third component in the solution and function as activating compounds. These compounds as well as oxygen influence the reduction of indigo carmine by cysteine.

We can make no concrete suggestion at this time concerning the impurity activating the solutions used by Dixon and Tunnicliffe.

The influence of physical factors. Agitation.

In order to secure quantitative data concerning the effect of agitation, 6 cc. of 0·1 *N* cysteine were added to eight electrode cells containing 100 cc. of phosphate buffer, p_H 7·4, and sufficient sodium hydroxide to neutralise the hydrochloric acid in the cysteine solution. 1 cc. of 0·02 *N* ferrous sulphate was added to electrode cells 1, 2, 3 and 4; 3 cc. of 0·02 *N* indigo carmine was added to all the electrode cells. The potential in each cell was approximately − 0·14 v. Five 2 cc. portions of 0·02 *N* hydrogen peroxide were added at intervals of about 20 minutes to electrode cells 3, 4, 7 and 8. During the addition of the peroxide the solution was agitated with a rapid stream of nitrogen passed through the solution. To electrode cells 1, 2, 5 and 6, 10 cc. of 0·02 *N* hydrogen peroxide were added at once and the agitation with nitrogen was stopped for 30 minutes. A distinct band of reduced indigo carmine formed in electrode cells 1 and 2 within a few minutes, but in cells 5 and 6 containing no ferrous sulphate the indigo carmine was reduced much more slowly. After the solutions in cells 1, 2, 5 and 6 were almost all reduced they were again agitated

with nitrogen and the velocity of oxidation of reduced indigo was then determined in all eight electrode cells. Since cells 1, 2, 3 and 4 contained iron, this experiment combines the influence of iron and that of agitation. The velocity of oxidation in cells 2, 4, 5 and 8 is shown in curves 2, 3, 4 and 5, respectively, in Fig. 1. It is assumed that the concentration of the third component is measured by the velocity of oxidation of the reduced indigo by the activated —SS grouping. That this is true will be shown presently. Since the concentration of the —SS grouping is the same in all cells, the difference in the velocity of oxidation must be due to the quantitative difference in the activation of the —SS grouping. It is apparent that iron has a great effect on the formation of the third component and that agitation does influence the reaction.

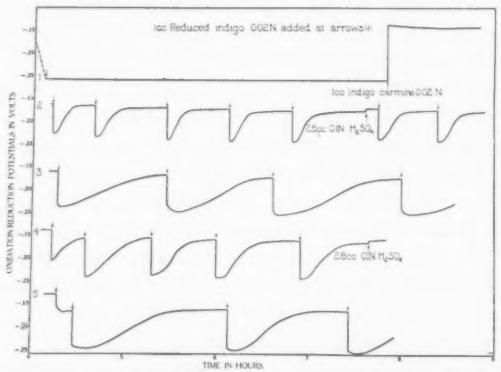
1cc Reduced indigo 002N added at arrow(1)
1cc Indigo carmine 0·02 N
0·5cc 0·1N H_2SO_4
0·5cc 0·1N H_2SO_4
OXIDATION REDUCTION POTENTIALS IN VOLTS
TIME IN HOURS

Fig. 1. The influence of iron and agitation on the formation of the third component.

Each of the solutions represented by curves 2, 3, 4 and 5 contained 6 cc. of 0·1 *N* cysteine in 100 cc. of phosphate buffer, p_H 7·4. Solutions 2 and 4 each contained 1 cc. of 0·02 *N* ferrous sulphate and 3 cc. of 0·02 *N* indigo carmine. 10 cc. of 0·02 *N* hydrogen peroxide were added in five 2 cc. portions to solution 3, while the solution was agitated with nitrogen. The same volume of peroxide was added to solution 2 without agitation. The difference in the velocity of oxidation of reduced indigo between solution 2 and solution 3 is due to the difference in the concentration of the third component. This difference is caused by agitation of the solution during the formation of the third component. Solutions 4 and 5 are similar to solutions 2 and 3, respectively, except that no ferrous sulphate was added to solutions 4 and 5. The only difference between solutions 4 and 5 is the factor of agitation, during formation of the third component. Solution 4 was not agitated. The difference between solutions 4 and 2, and 5 and 3, respectively, shows the influence of increased iron content. Solution 1 contained 1 cc. of 0·02 *N* reduced indigo only and the steady potential is good evidence that oxygen was not present in the nitrogen.

Since cysteine alone will reduce hydrogen peroxide without the formation of the third component, and since the peroxide must first react with the indigo carmine in order to form the third component, it is evident that two competing reactions are going on simultaneously in the solution. Any influence, therefore, which can increase the amount of peroxide reacting with the indigo carmine and prevent its interacting first with the —SH group will increase the concentration of the third component in the solution. Iron is one factor increasing the velocity of interaction between hydrogen peroxide and indigo carmine. If agitation decreases the amount of peroxide interacting with indigo carmine this would appear to be the probable mechanism by which

agitation inhibits the formation of the third component. Whether or not this is the correct explanation does not alter the fact that agitation does modify in a significant manner the formation of the third component in these solutions.

Not only does the formation of the essential third component depend on agitation but the concentration of the hydrogen peroxide added is also of importance; 2 cc. of 0·1 *N* hydrogen peroxide do not result in as high a concentration of the third component as 10 cc. of 0·02 *N* hydrogen peroxide; furthermore, Nord [1927] has shown that the activating compound is thermolabile and a solution which is capable of reducing indigo carmine when prepared as already outlined is found to have lost its power to reduce indigo carmine if the solution is heated to boiling for 5 minutes. The boiling must be carried out in the complete absence of oxygen. After cooling to the temperature of the thermostat such a solution cannot reduce more than traces of 0·02 *N* indigo carmine, but it can be reactivated by the subsequent addition of oxygen.

In this experiment the assumption was made that the velocity of oxidation of reduced indigo is proportional to the concentration of the third component in the solution. In order to show that this assumption is correct eight electrode cells were prepared with the same amounts, 3 cc. 0·1 *N* cysteine and 3 cc. 0·1 *N* cystine in 100 cc. phosphate buffer, p_H 7·4. To these solutions the following amounts of a solution containing the third component were added: cell 1, 0 cc., cell 2, 3 cc., cell 3, 6 cc., cell 4, 9 cc., cell 5, 12 cc., cell 6, 18 cc., cell 7, 24 cc. and cell 8, 30 cc. The preparation of the solutions is given on p. 663. Fig. 2 shows the velocity of oxidation of 1 cc. of 0·02 *N* reduced indigo in cells 1, 2, 3, 4, 5, 7 and 8. The curves are good evidence that the concentration of the third component is the determining factor in the velocity of oxidation of reduced indigo with cystine.

Dixon and Tunnicliffe confirmed the statement made by Kendall and Nord that indigo carmine is more rapidly reduced by cysteine if hydrogen peroxide is added to the solution; however, since they were able to reduce indigo carmine without the addition of oxygen or hydrogen peroxide the accelerating effect of hydrogen peroxide was considered by them as of little significance. They point out that the action of hydrogen peroxide may be either on the cysteine or on the indigo carmine. In order to determine which of these compounds was affected by the peroxide they prepared a solution of indigo carmine treated with dilute hydrogen peroxide. The excess peroxide was destroyed with manganese dioxide and it was then found that this solution, filtered free from the manganese dioxide, accelerated the reduction of indigo carmine. They state that this result shows conclusively that by the action of hydrogen peroxide on indigo carmine products are formed which have a marked catalytic effect on the reduction of indigo carmine by cysteine, and they say, "we consider that this fact offers the full explanation of the phenomenon observed by Kendall and Nord and renders unnecessary the assumption of the formation of the active oxygen addition product with cysteine."

We have repeated Dixon and Tunnicliffe's treatment of indigo carmine with hydrogen peroxide, removed the excess peroxide with manganese dioxide, and have confirmed their observation that this solution accelerates the reduction of indigo carmine. However, we have performed two other experiments with this solution and the result of this work emphasises the necessity for the assumption of something in the solution other than an oxidation product of indigo carmine.

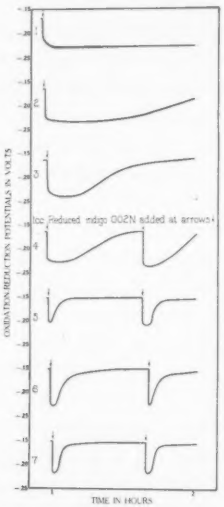

Fig. 2. The influence of the concentration of the third component on the velocity of oxidation of reduced indigo by seven solutions each containing 100 cc. phosphate buffer, p_H 7·4, 3 cc. of 0·1 *N* cysteine and 3 cc. of 0·1 *N* cystine.

0, 3, 6, 9, 12, 24, 30 cc. of a solution containing the third component were added to the solutions 1, 2, 3, 4, 5, 6 and 7 respectively. The increased velocity of oxidation is due to activation of the —SS grouping by the third component. Although not shown in the figure the velocity of oxidation of reduced indigo by 30 cc. of the solution containing the third component alone was not greater than that shown in solution 3. The preparation of the solution of the third component is given on p. 663.

The first experiment was to allow the oxidation of 0·02 *N* indigo carmine with an equal volume of 3 % hydrogen peroxide and 1 cc. of 0·02 *N* ferrous sulphate to proceed until the solution was entirely bleached. The peroxide was then destroyed with manganese dioxide and 6 cc. of this solution were added to 100 cc. of phosphate buffer, p_H 7·4, containing 6 cc. of 0·1 *N* cysteine and 3 cc. of 0·02 *N* indigo carmine. The bleached indigo carmine solution did not permit the reduction of the indigo carmine by the cysteine. The experiment was repeated and the results are good evidence for the fact that the accelerating action of hydrogen peroxide and indigo carmine is not due to oxidised indigo carmine alone.

The second experiment which bears on this point was performed as follows. 0·02 *N* indigo carmine was bleached with an equal volume of 3 % hydrogen peroxide and 1 cc. of 0·02 *N* ferrous sulphate until one-half of the indigo carmine had been destroyed. The extent of bleaching of the indigo carmine

was determined by diluting the solution and matching in a colorimeter, using the original solution suitably diluted as a standard. The remaining hydrogen peroxide was then destroyed with manganese dioxide and it was shown that the filtrate was able to catalyse the reduction of indigo carmine by cysteine. A portion of this solution was boiled after the excess of hydrogen peroxide had been removed and before it was deoxygenated. After boiling, which had produced no change in the colour of the solution, it was found that cysteine could no longer reduce the indigo carmine. This result is in agreement with the experiment already described in which the third component was destroyed by boiling a solution containing cysteine, cystine and the third component, and is evidence that some thermolabile intermediate product is the essential agent which activates the —SH group. This will be discussed somewhat more in detail when the effect of sodium disulphide is described, and in the last section of this paper.

We have also confirmed Dixon and Tunnicliffe's observation that iron greatly accelerates the action of hydrogen peroxide on indigo carmine. It was interesting to note that the indigo carmine which we used was so free from iron that 0·02 *N* hydrogen peroxide had no bleaching power and when the 0·02 *N* indigo carmine solution was mixed with an equal volume of 3 % hydrogen peroxide it reacted so slowly that it could stand 20 hours without destruction of more than 50 % of the indigo carmine.

The rôle of sodium disulphide in the formation of the addition compound.

It is difficult to reply to Dixon and Tunnicliffe's comments on the action of sodium disulphide since their reference to the paper of Kendall and Nord is so contrary to the statements made in that paper. Through an oversight in the preparation of the manuscript the method used in the preparation of sodium disulphide was omitted, but, since there is only one way by which this compound can be prepared readily, this omission was not misleading. The sodium disulphide was prepared by the addition of sulphur to a solution of sodium sulphide. The method used, in brief, was as follows. A solution containing 40 % of sodium hydroxide was saturated with hydrogen sulphide. The solution was then diluted to such an extent that when it was cooled crystals of sodium sulphide separated in large flat plates on the bottom of the container. If the solution was too concentrated the crystals separate in a solid mass. The solution was diluted so that but a small percentage of the sodium sulphide separated. The crystals were separated from the solution by decantation and were washed with a small amount of distilled water which was immediately decanted.

10 g. of crystals were then weighed roughly on a balance and dissolved in 100 cc. of water. This solution was then titrated for its sodium sulphide content with 0·1 *N* iodine. It was then made to 0·7 *N*. To 50 cc. of the 0·7 *N* sodium sulphide solution the equivalent amount of flowers of sulphur was

added. The sulphur dissolved readily, forming a yellow solution; this was diluted with distilled water until it was 0·01 *N*.

The statement of Dixon and Tunnicliffe that sodium disulphide is a powerful reducing agent requires more definite description of the conditions used in determining its reducing action. A solution of 0·01 *N* sodium disulphide added to 100 cc. of buffer, p_H 7·4, immediately hydrolyses with the precipitation of colloidal sulphur and liberation of hydrogen sulphide. Although hydrogen sulphide is a reducing agent capable of reducing indigo carmine, sulphur is an oxidising agent capable of oxidising reduced indigo carmine. If oxygen-free nitrogen is passed through the solution the hydrogen sulphide is readily removed and the colloidal sulphur is left in suspension.

If indigo carmine is present in the solution when the sodium disulphide is added, slight, and only slight, reducing action is evident. 1 cc. of 0·02 *N* indigo carmine in 100 cc. of phosphate buffer, p_H 7·4, is not reduced by five times its equivalent of sodium disulphide; on the other hand, the precipitated sulphur is an oxidising agent when a sufficiently sensitive criterion is used, namely, reduced indigo or reduced indigo carmine. The precipitated sulphur will oxidise its full equivalent of reduced indigo, and after each addition of reduced indigo the potential will return to the value of approximately − 0·14 v. It was stated by Kendall and Nord [1926, p. 320] that "feeble ability to reduce indigo carmine and approach the same equilibrium point" was also shown, that is − 0·14 v.

If, however, the reducing power of sodium disulphide is tested in distilled water instead of phosphate buffer of p_H 7·4, the higher hydroxyl concentration prevents hydrolysis and colloidal sulphur does not separate. The solution ceases to have oxidising power and under these conditions it is, as Dixon and Tunnicliffe say, "a most powerful reducing agent."

The two statements of Dixon and Tunnicliffe, first, that sodium disulphide is a powerful reducing agent in the condition in which it was used by Kendall and Nord, and second, that no control experiments with sodium disulphide alone were made, are both incorrect. Fig. 8 in the paper by Kendall and Nord is devoted entirely to the oxidising action of sodium disulphide, and the effect on indigo carmine is clearly stated. However, even though sodium disulphide was a powerful reducing agent at p_H 7·4 and could reduce its full equivalent of indigo carmine, still the explanation of the reduction of indigo carmine given by Dixon and Tunnicliffe is impossible since the amount of sodium disulphide added was in each case less than the amount of indigo carmine used.

Since sodium disulphide cannot reduce indigo carmine at p_H 7·4, the reduction of this dye with cysteine and sodium disulphide cannot be explained except on the basis of activation of the —SH group.

It has now been found that colloidal sulphur prepared by acidifying sodium disulphide with its equivalent of sulphuric acid and completely removing the hydrogen sulphide is also capable of activating the sulphydryl group so that it can reduce indigo carmine. Iron does not increase or prevent the activating action of sodium disulphide or colloidal sulphur.

Colloidal sulphur is not a sufficiently powerful oxidising agent to oxidise indigo carmine in the same way as does hydrogen peroxide. The effect cannot be solely on indigo carmine and must involve the sulphydryl group. It is for this reason that it seemed most probable that the interaction of hydrogen peroxide and indigo carmine does not involve, primarily and only, the indigo carmine; its effect on the sulphydryl group is due to a definite change in the intensity relations within the sulphydryl group itself, the result of which is to enable it to enter into chemical reactions and to exert reducing action which cannot be demonstrated in the absence of suitable activation.

Control of hydrogen ion concentration.

The criticism of the control of hydrogen ion concentration does not appear to be well taken. The acidity or alkalinity of all solutions prior to their introduction into the electrode chambers was determined by titration with 0·1 *N* acid or alkali, using phenol red as indicator. If 6 cc. of the sodium salt of cystine were added to the solution, 6 cc. of deoxygenated 0·1 *N* sulphuric acid were subsequently added and, although it was not stated in the paper, the hydrogen ion concentration of the solution was determined by colorimetric comparison after this procedure and it was found that the p_H of the solution was not affected. If the hydrochloride of cysteine was used, an equivalent amount of deoxygenated sodium hydroxide was added to the solution in some cases; in other cases a definite amount of hydrochloric acid was left unneutralised, but, if so, the same known amount of acid was present in all eight electrode cells used in each experiment. Fig. 3 in the paper of Kendall and Nord shows the influence of adding 6 cc. of 0·1 *N* sulphuric acid to the solution and such a change in the p_H does not alter the characteristic poising action of the solution and in no way alters the conclusions drawn. The charts and statements included in the paper of Kendall and Nord were selected from a large mass of data and were chosen to show definite influences on the —SH and —SS groupings. Many controls and variations in the technic were carried out but could not be included. Dixon and Tunnicliffe give no experimental results to show that in any way the procedure followed by Kendall and Nord resulted in lack of control of the hydrogen ion concentration, and the procedure followed was stated by Kendall and Nord in detail sufficient to permit duplication of the results.

The use of indigo reduced with glucose and alkali.

Dixon and Tunnicliffe state that the method of preparation of solutions of reduced indigo which Kendall and Nord used in experiments on the reversibility of —SH and —SS systems seems also to call for some notice, and finally that "it is obvious that such a solution containing one of the most powerful reducing agents, glucose, in the presence of alkali together with the unknown decomposition product of glucose will give potentiometric readings of little significance."

Before the solution of reduced indigo was used for this work careful controls were carried out concerning the quantitative value of its reduction potentials. The action of alkali and glucose causes the complete solution of all the indigo added to the solution and, provided it is a preparation of synthetic indigo of high purity, the concentration of the reduced indigo in solution is equivalent to the quantity used. 1 cc. of 0·02 *N* reduced indigo solution, prepared as previously described, by the use of glucose and sodium hydroxide, added to 100 cc. of oxygen-free phosphate buffer, p_H 7·4, will produce a reduction potential equivalent to approximately − 0·26 to − 0·28 v. If to this 1 cc. of 0·02 *N* solution of an oxidising dye is added, for example, indigo carmine, methylene blue or dibromophenolindophenol, the potential drops immediately to a point depending on the E_0 value of the oxidising dye. As soon as all the reduced indigo is oxidised with indigo carmine the potential decreases at least 0·15 v., and with the other oxidising dyes the drop in potential after complete oxidation of the reduced indigo may be as great as 0·3 v. Instead of a solution containing "one of the most powerful reducing agents" as stated by Dixon and Tunnicliffe, actual observations show that the maximal reduction potential of such a solution, except for the reduced indigo, is not more than − 0·12 v. This may be readily shown by preparing such a solution using all the constituents except the indigo; in other words, when 4·5 g. of glucose is boiled with 45 cc. of *N* sodium hydroxide and 250 cc. of water, through which oxygen-free hydrogen is passed, and the boiling is continued for 5 minutes after the last addition of the glucose to the alkali, the maximal reduction intensity of this solution is not greater than − 0·12 v.

Other evidence showing conclusively that the reduced indigo solution, prepared with glucose, contains no substance which can affect the quantitative values in potentiometric titrations, as it was used, is afforded by titration of the reduced indigo with indigo carmine, the reduced indigo being added to 10 cc. of indigo carmine in the electrode cell. It is apparent that, by adding the reduced indigo to the dye, all substances in the solution which can be oxidised at an intensity equal to that of the dye will cause a reduction of indigo carmine. If, then, the reverse procedure is carried out, the indigo carmine being added to 10 cc. of the reduced indigo, the high reducing power of the reduced indigo will remove all oxidising power of the indigo carmine before the indigo carmine can oxidise reducing substances in the solution of less reducing intensity than reduced indigo. The addition of indigo carmine is stopped when the potential of − 0·16 v. indicates complete oxidation of the reduced indigo. When such a technic is followed it is found that the indicated strength of the reduced indigo is identical by both methods. If anything other than reduced indigo were present which could be oxidised with indigo carmine this relationship could not hold. When the same experiment is tried with dibromophenolindophenol it is found that less reduced indigo solution is required when it is added to 10 cc. of the dibromo-dye. This is evidence that, if the maximal oxidation potential of the dye is greater

than approximately − 0·12 v., substances in the reduced indigo solution formed by the action of alkali on the glucose will reduce some of the dye. This fact does not affect the accuracy of the method as used for solutions of cystine.

Since the maximal oxidising intensity of the —SS grouping in the presence of reduced indigo carmine is less than the maximal reducing intensity of the glucose solution by at least 0·05 v., it is obvious that there is no reducing compound in the glucose solution which can be oxidised by the —SS grouping; on the other hand, the entire amount of reduced indigo can be oxidised by the —SS grouping at a level of intensity above the maximal reduction intensity of the other constituents of the glucose solution.

10 cc. of 0·02 *N* reduced indigo in 100 cc. of phosphate buffer, p_H 7·4, containing 0·1 *N* acid, just sufficient to bring the p_H of the reduced indigo to 7·4, can be accurately titrated with 0·02 *N* dibromophenolindophenol, 0·02 *N* methylene blue or 0·02 *N* indigo carmine. The volume of oxidising dye required to decrease the potential to a value − 0·16 v. (indicating the complete oxidation of the reduced indigo) is the same with these three dyes, and affords an accurate assay of the indigo in the solution.

Whether or not the formation of products from the oxidation of glucose affects the reaction is another matter; such an effect has been shown on oxidation of adrenaline and related compounds, but after using solutions of reduced indigo for 3 years we have observed no such effect on the —SH grouping and the solution has justified its use in many ways. Control blanks on the stability have shown that it is stable in the phosphate buffer solution for many hours, and the results which we have obtained on the oxidising power of the —SS group and the results published by Kendall and Nord cannot be lightly dismissed.

Another point which should be emphasised is the fact that all of these solutions were compared on a relative basis; if a series of eight electrode cells containing various solutions were titrated with reduced indigo it was the differences in the velocity of oxidation which indicated the activity of the —SS grouping. The potential following the addition of 1 cc. of 0·02 *N* reduced indigo to 100 cc. of phosphate buffer, p_H 7·4, is shown in curve 1, Fig. 1. Oxygen-free nitrogen was passed through the solution for 4 hours without significant change in the potential. 1 cc. of 0·02 *N* indigo carmine oxidised the reduced indigo and produced an immediate drop in potential to − 0·15 v. Similar evidence that the nitrogen used by Kendall and Nord was free from oxygen is given in Fig. 9 of their paper. 3 cc. of 0·02 *N* reduced indigo was not oxidised by passing nitrogen through the solution for 6 hours.

All of the conclusions of Kendall and Nord were based on the relative velocity of the change in potential and were not concerned with the total volume of reduced indigo which was oxidised. For a study of such reactions it is imperative that the reducing agent used be a substance of such a nature that the oxidised form produced by the reaction will not accumulate in the

solution and thereby influence the equilibrium point of the solution. Reduced indigo is such a substance and the criticisms of Dixon and Tunnicliffe, although applicable in the presence of a stronger oxidising agent than the —SS grouping, do not affect the value or interpretation of the results obtained with this reagent.

It is difficult to understand Dixon and Tunnicliffe's criticism of the alkalinity of the reduced indigo solution. Instead of stating that neutralisation of reduced indigo was unnecessary, Kendall and Nord included in the Figs. 1, 2, 3 and 4 the amount and time when the acid was added to compensate for the alkalinity of the reduced indigo added; the letter "J" was used to denote the addition of acid to the solution. The statement of Dixon and Tunnicliffe, that the alkalinity cannot be determined by an indicator, has no bearing on the procedure followed. They state that the addition of 40 % caustic soda failed to change the colour of phenol red added to the solution after removal of the indigo. This statement is true but is without significance, as the addition of only 2 to 3 cc. of 0·1 *N* sulphuric acid to 5 cc. of the reduced indigo solution after removal of the indigo will change the indicator in a satisfactory manner and indicate the amount of sodium hydroxide present. They estimated that in a sample of reduced indigo which they prepared the p_H was approximately 12; this hydroxyl ion concentration is less than that of a 0·1 *N* solution of alkali; therefore, for each 5 cc. of reduced indigo, less than 5 cc. of oxygen-free 0·1 *N* sulphuric acid would be required to make the resultant p_H in the solution the same as before the addition of the reduced indigo solution.

From the first use of reduced indigo it has been the practice in this laboratory to determine the alkalinity of each preparation. As ordinarily prepared, 5 cc. of such a solution requires between 2·0 and 3·0 cc. of 0·1 *N* sulphuric acid to bring it to neutrality, using phenol red as indicator; this amount of acid has been added after the addition of each 5 cc. of reduced indigo. No error can result from this procedure which can in any way alter the conclusions concerning the oxidising power of the —SS grouping. A quantitative measure of the effect of the slight alkalinity resulting from the addition of 5 cc. of reduced indigo solution is shown in Fig. 1. At 7 hours 37 minutes 2·8 cc. of 0·1 *N* sulphuric acid were added to the solutions in cells 2 and 3. The reduction potential of the solution had been increased 5 millivolts by the 5 cc. of reduced indigo above the potential indicated at 4 hours 35 minutes. Addition of the sulphuric acid again brought the potential to the precise value shown 3 hours previously, although the equilibrium potential had been displaced four times during this interval by an amount equal to more than twenty times the effect of the alkali in the reduced indigo. Kendall and Nord submitted evidence to show that in a solution containing an activating agent the —SS grouping is capable of acting as an oxidising agent. The paper of Dixon and Tunnicliffe does not show that the —SS grouping cannot act in this manner.

The influence of the —SH/—SS ratio.

Dixon and Tunnicliffe state that examination of the experimental evidence of Kendall and Nord fails to establish the conclusion that it is the ratio of the —SH to the —SS grouping which determines the potential in solutions containing the activating component. The potentiometric evidence for this statement will be given in another paper; however, there is other evidence which has a direct bearing on this point. If 25 cc. of 0·1 *N* cysteine hydrochloride and 2 cc. of 0·02 *N* ferrous sulphate are placed in 162 cc. of phosphate buffer, p_H 7·4 (with sufficient sodium hydroxide to neutralise the hydrochloric acid), in a 500 cc. flask containing an inlet tube, a delivery tube, and an outlet on the side of the neck, and the solution is then deoxygenated, 12·5 cc. of oxygen-free 0·02 *N* indigo carmine can be added through the side tube on the neck without reduction of the indigo carmine. If to such a solution 37·5 cc. of 0·02 *N* hydrogen peroxide are added without agitation the indigo carmine is promptly reduced. Before and after the introduction of the hydrogen peroxide, oxygen-free nitrogen is passed through the flask and out through the tube on the neck. The nitrogen is purified in a special small copper furnace and the gas passes through nothing except copper tubing or glass after it has been freed from oxygen. The third component is produced in the solution and the indigo carmine is completely reduced to a canary yellow colour. This solution is transferred to a movable burette, similar to one described by Clark and Cohen, and 30 cc. are placed in each of eight electrode cells. The colour is uniform. If to six of this series of cells 1 cc. of 0·1 *N* cysteine and 1, 2, 3, 4, 5, 6 cc. of 0·1 *N* cystine (as the sodium salt) and the corresponding amount of oxygen-free 0·1 *N* sulphuric acid are added, a series of solutions will be prepared in which the ratio of the —SH to the —SS grouping varies within wide limits. The amount of cysteine and the p_H are the same in all. Such a series of solutions shows a perfect gradation in the potential of the equilibrium point; moreover, it shows a corresponding change in the colour of the indigo carmine. The added cystine changes the —SH/—SS ratio and this in turn changes the ratio of the reductant/oxidant forms of indigo carmine.

A comparison of the colours of the solution is good evidence that the oxidation-reduction intensity in the solution is determined by the —SH/—SS ratio. If to one of the remaining two cells, 3 cc. of 0·1 *N* cysteine are added, there will be an increase in the reduction potential and a still further intensifying of the canary yellow colour resulting from the reduction of the indigo carmine. If 1 cc. of 0·1 *N* cystine but no cysteine is added to the remaining cell, the potential is decreased more than in cell 1. The colour corresponds. Not only is the colour of the solution determined by the ratio —SH/—SS, but the velocity of oxidation of the reduced indigo is tremendously accelerated in those solutions containing the higher concentrations of the —SS grouping. Further evidence concerning this will be given in a paper on the potentiometric measurements.

ACTIVATION OF —SH AND —SS GROUPINGS.

The work described in the preceding part of this paper furnishes conclusive evidence that the velocity of oxidation of cysteine is influenced by other constituents in the solution which have the power to activate the sulphydryl group. In order further to study the effect of oxygen on this reaction an apparatus similar to the one described by Dixon and Tunnicliffe was constructed, the form of which is shown in Fig. 3. No rubber stoppers were used but the two openings were closed with ground glass tubing to which glass stopcocks were fused. The apparatus consisted of four compartments which made it possible to add activating substances, such as hydrogen peroxide, sodium disulphide or colloidal sulphur on one side only. One half always served as a check for the other. By the use of a high vacuum pump capable of producing a vacuum equivalent to a fraction of a millimetre of

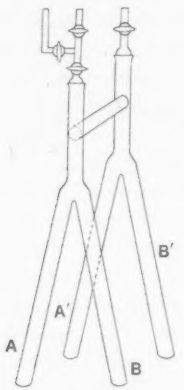

Fig. 3.

mercury, not only were oxygen and nitrogen removed, but the water vapour formed by the rapid evaporation of about one-third of the solution carried out from the apparatus all traces of oxygen which could be removed by this means. Nitrogen was admitted and the apparatus was again evacuated. This was repeated three times, and the experiment was then carried out in a vacuum. The amounts of cysteine, indigo carmine and activating solutions used are given in Table I. In one side of the apparatus, compartments A, A', were placed the buffer, cysteine and one-half of the water; on the other side, compartments B, B', were placed the ferrous sulphate, indigo carmine, one-half of the water and the activating agent. When the apparatus was tipped A' mixed with B', A with B. The volume given in the table shows the volume of each constituent after mixing. The sodium hydroxide is given in terms of normal solution but where the volume was less than 0·5 cc., 0·1 N solution was used. All solutions which did not reduce in the time given in the chart were immediately activated by the addition of hydrogen peroxide and a small amount

of iron. In each case the indigo carmine was reduced following this treatment. The last 13 experiments were performed with a solution of the same composition in each of the four compartments. The solutions which were present at the same time in the apparatus are indicated in the Table by *A* and *B*.

Table I. *The time required for reduction of indigo carmine by activated cysteine.*

	0·1 *N* cysteine, cc.	0·1 *M* ferrous sulphate, cc.	*N* sodium hydroxide, cc.	Phosphate buffer, p_H 7·6, cc.	Water, cc.	0·2 *N* indigo carmine, cc.	0·2 *N* hydrogen peroxide, cc.	0·2 *N* colloidal sulphur, cc.	0·01 *N* sodium disulphide, cc.	0·01 *M* adrenalone, cc.	Time required for reduction
A	0·5	0·1	0·17	3·0	10·0	0·5	—	—	—	—	Not in 18 hrs.
B	0·5	0·1	0·17	3·0	10·0	0·5	1·0	—	—	—	20 mins.
A	1·0	0·1	0·35	3·0	10·0	0·5	1·0	—	—	—	20 mins.
B	1·5	0·1	0·52	3·0	10·0	0·5	1·0	—	—	—	15 mins.
A	2·0	0·1	0·70	3·0	10·0	0·5	1·0	—	—	—	10 mins.
B	2·5	0·1	0·875	3·0	10·0	0·5	1·0	—	—	—	10 mins.
A	1·5	0·1	0·52	3·0	10·0	0·5	—	—	1·0	—	10 mins.
B	0·0	0·1	—	3·0	10·0	0·5	—	—	1·0	—	Not reduced
A	1·5	0·1	0·52	3·0	10·0	0·5	—	2·0	—	—	5 mins.
B	0·0	0·1	—	3·0	10·0	0·5	—	2·0	—	—	Not reduced
A	1·5	—	0·52	6·0	5·0	0·5	—	—	1·0	—	5 mins.
B	1·5	—	0·52	6·0	5·0	0·5	—	—	—	—	Not in 60 mins.
A	2·5	—	0·87	3·0	10·0	0·5	—	—	—	2·27	75 mins.
B	2·5	—	0·87	3·0	10·0	0·5	—	—	—	0·45	90 mins.
	0·0	—	—	3·0	10·0	0·5	—	—	—	0·45	Not in 105 mins.
	2·5	0·1	0·87	3·0	10·0	0·5	—	—	—	—	16 hrs.
A	1·5	—	0·52	6·0	5·0	0·5	—	—	—	—	8 hrs.
B	2·5	0·1	0·87	3·0	10·0	0·5	—	—	—	—	2½ hrs.
	2·5	0·1	0·87	3·0	14·0*	0·5	1·0	—	—	—	7 mins.
A	2·5	0·5	0·83	3·0	5·0	1·0	0·5	—	—	—	15 mins.
B	2·5	—	0·83	6·0	10·0	1·0	0·5	—	—	—	2 hrs.
A	2·5	0·1	0·87	3·0	14·0*	0·5	1·0	—	—	—	5 mins.
b	2·5	0·1	0·87	3·0	14·0*	0·5	—	—	—	—	Not in 5 hrs.
A	2·5	0·1	0·87	3·0	10·0	0·5	—	—	—	—	Not in 4 hrs.
B	2·5	0·1	0·87	3·0	10·0	0·5	—	—	—	—	80 mins.
	2·5	—	0·83	6·0	10·0	1·5	—	—	—	—	Not in 10 hrs. 20 mins.
A	2·5	0·1	0·87	3·0	10·0	0·5	—	—	—	—	Not in 12 hrs.
B	2·5	0·1	0·87	3·0	10·0	0·5	—	—	—	—	9 hrs.
A	1·5	0·1	0·52	3·0	10·0	0·5	—	—	—	—	9 hrs.
B	1·5	0·1	0·52	3·0	10·0	0·5	—	—	—	—	7½ hrs.
	2·5	—	0·87	3·0	10·0	0·5	—	—	—	—	1 hr.
	5·0	—	1·66	3·0	20·0	1·0	—	—	—	—	All four compartments in 45 mins.
	1·3	—	1·1	1·6	5·5	0·3	—	—	—	—	5 hrs. 25 mins.
	1·3	—	1·1	1·6	5·5	0·3	—	—	—	—	6 hrs. 10 mins.
	1·3	—	1·1	1·6	5·5	0·3	—	—	—	—	6 hrs. 10 mins.
	1·3	—	1·1	1·6	5·5	0·3	—	—	—	—	6 hrs. 25 mins.
	1·5	—	0·5	1·8	6·0	0·3	—	—	—	—	2 hrs. 30 mins.
	1·5	—	0·5	1·8	6·0	0·3	—	—	—	—	5 hrs.
	1·5	—	0·5	1·8	6·0	0·3	—	—	—	—	8 hrs. 45 mins.
	1·5	—	0·5	1·8	6·0	0·3	—	—	—	—	9 hrs. 10 mins.
	2·1	—	0·7	2·5	4·2	0·4	—	—	—	—	2 hrs. 30 mins.
	2·1	—	0·7	2·5	4·2	0·4	—	—	—	—	2 hrs. 50 mins.
	2·1	—	0·7	2·5	4·2	0·4	—	—	—	—	3 hrs. 15 mins.
	2·1	—	0·7	2·5	4·2	0·4	—	—	—	—	3 hrs. 20 mins.

* 4 cc. of water evaporated while in a vacuum.

The difference in the velocity of reduction was striking and was conclusive evidence that the activity of the —SH group was tremendously affected by the presence of the third component formed in the solution. However, in no

case did the indigo carmine in the unactivated solution remain in the oxidised form for more than a few hours.

The original experiments in which it was shown that cysteine cannot reduce indigo carmine were again carried out in electrode vessels through which oxygen-free nitrogen was continuously passed. Although the phosphate buffer, cysteine and indigo carmine were present in the same amounts as were used in the vacuum apparatus, the indigo carmine remained in the oxidised form for 72 hours. Furthermore, when this solution was prepared in the movable burette, which was used to add solutions to the electrode chambers, it was found that the indigo carmine remained in the oxidised form for more than 48 hours. A solution containing the indigo carmine and a second solution containing the cysteine, after being thoroughly deoxygenated, were mixed in an electrode cell which had been deoxygenated; the solution was then passed back into the movable burette and further deoxygenated. After this treatment the indigo carmine remained in the oxidised form although no nitrogen was passed through the solution. When air or hydrogen peroxide was admitted to these solutions, which had remained for from 48 to 72 hours unchanged, the indigo carmine was completely reduced within 5 minutes.

After confirmation of the observation that indigo carmine is not reduced by cysteine in the absence of oxygen when the solutions are held in electrode cells or in the movable burette, further experiments were made to determine the time required to reduce the indigo carmine by cysteine in the vacuum apparatus. The significant observation was then made that the time required for the reduction of indigo carmine varied greatly for the same solution in the four different compartments; the difference was sometimes as great as 6 hours. Furthermore, the indigo carmine in small drops of the solution which adhered to the cross tube of the apparatus was always promptly reduced. These observations were explained by the assumption that, even though the apparatus had been held in a high vacuum for several hours, sufficient oxygen remained adsorbed on the surface of the glass to bring about prompt reduction in a small volume of the solution and that the varying velocity of reduction in the four compartments was due to the varying amounts of oxygen adsorbed on the glass.

One further series of experiments was then made in order to remove all oxygen from the surface of the apparatus. A solution of glucose, to which sodium hydroxide and indigo carmine were added, was placed in the apparatus, completely filling all parts, including a short section of rubber tubing attached to the inlet tube. The alkaline glucose reduced the indigo carmine and, after this powerful reducing agent had remained in contact with the glass for a few hours, the apparatus was thoroughly washed with distilled water which had been previously deoxygenated with oxygen-free nitrogen. The water was added to the apparatus through the movable burette. After several washings all traces of alkaline glucose were removed and, through the use of the same

movable burette, it was possible to add the mixture of indigo carmine and cysteine without exposure to oxygen. At no time was any part of the apparatus except the rubber inlet tube exposed to air during the deoxygenation and subsequent washing out of the reducing solution. The solution containing cysteine-indigo carmine was equally divided in the four arms of the apparatus which was then evacuated. It was observed that the time required for the reduction of the indigo carmine was greatly extended, but still complete reduction occurred after about 9 hours. It then became evident that oxygen cannot be sufficiently excluded by this type of experiment to establish the fact that cysteine cannot reduce indigo carmine in the complete absence of oxygen.

During this work it was noted that if a trace of vaseline remained on the surface of the glass it was impossible to remove the oxygen from the glass and vaseline. On one occasion a small particle of vaseline broke off from the glass stopcock and floated on the surface of the solution. This was the focus from which the activation of the solution could be followed and prompt reduction of the indigo carmine occurred in an ever-widening circle surrounding this minute source of oxygen. These results show that when an apparatus of this type is used the conclusion that cysteine can reduce indigo carmine in the complete absence of oxygen is not justified. The activation of a solution containing cysteine and indigo carmine is such a delicate reaction that it will serve as a test for the minutest traces of oxygen.

It doubtless is possible to prepare an apparatus similar to the one described in which indigo carmine will remain in the oxidised form for 72 hours, but the extreme precautions which would have to be used would probably require fused glass connections throughout. These obstacles have prevented our carrying out this experiment and since the crucial experiment is furnished by the use of the moveable burette as well as the electrode cell, we regard these results as sufficiently conclusive to justify the statement that in the complete absence of oxygen and other activating agents cysteine cannot reduce indigo carmine.

From these results we conclude that hydrogen does not dissociate from cysteine with the formation of cystine except in the presence of compounds which can suitably interact with the sulphydryl group. Interaction with the sulphydryl grouping may be brought about by agents other than indigo carmine and oxygen, such as colloidal sulphur and possibly substances which may occur as impurities in cysteine or indigo carmine.

Summary.

Cysteine cannot reduce indigo carmine and cystine cannot oxidise reduced indigo carmine or reduced indigo in the absence of a third component which functions as an activating agent. This activating agent is produced through the action of oxygen or sodium disulphide on indigo carmine.

Iron cannot activate the —SH or —SS grouping, but iron markedly influences the velocity of reaction between oxygen and indigo carmine. This results in an increased concentration of the activating agent. Agitation of the solution and other physical factors influence the formation of the activating agent. It is destroyed by heat. Iron neither accelerates nor prevents the action of sodium disulphide on indigo carmine.

The chemical nature of the activating agent appears to be an unstable oxygen or sulphur addition product of indigo carmine, and its essential action involves the activation of the sulphur atom, which permits the sulphydryl and disulphide groupings to manifest their latent reducing or oxidising power. In the presence of a constant concentration of the activating agent the velocity of oxidation and reduction is determined by the ratio C.SH/C.SS.C groupings.

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LXXXII. THE MECHANISM OF OXIDATION-REDUCTION POTENTIAL.

I. THE OXIDATION-REDUCTION POTENTIAL OF CYSTEINE AND CYSTINE.

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In a recent paper Dixon [1927] has discussed the mechanism of the reduction potential. He points out that the theory of Wieland concerning the transfer of hydrogen from reductant to oxidant can be used to deduce the equilibrium equations. On the other hand these equations have been derived by Clark [1923], who assumes that the phenomenon may be due to electron transfer and that the reduction mechanism does not necessarily involve the actual transfer of hydrogen. The viewpoint of Dixon appears to have complicated rather than simplified the problem of oxidation-reduction phenomena. Whether the potential is due to transfer of hydrogen or electrons will not be discussed, but we wish to reply to another portion of the paper in which Dixon takes up as a specific instance the mechanism of the reduction potential of cysteine. He assumes that hydrogen which is adsorbed on the surface of the platinum is derived from three sources: hydrogen gas, hydrogen ion and hydrogen dissociating from a reductant. He assumes that the portion of the hydrogen on the surface of the platinum diffusing away as hydrogen gas is an unimportant factor in those cases where prompt equilibrium is attained between the reductant and the platinum, but that the reduction potential of cysteine is determined by a "steady state" which is a kinetic equilibrium between two processes; dissociation of hydrogen from the reductant, R.C.SH, and the diffusion of hydrogen gas away from the platinum electrode. The mechanism suggested which postulates a "steady state" forces the addition of three corollaries to the present conception of the mechanism of reduction: (1) that diffusion of hydrogen away from the platinum electrode is of such importance that it actually determines the reduction potential of at least one reductant, cysteine; (2) that not merely does the electrode register the oxidation-reduction potential of the solution but that a kinetic equilibrium is established on the surface of the metal electrode which determines the equilibrium potential, and (3) that cysteine has unique reducing power

which differs from that of other hydrogen donators in that the oxidant of cysteine has no effect on the platinum electrode. We submit evidence in this paper to show that these three assumptions are incorrect and that the postulation of a compensating process of hydrogen diffusion is unnecessary to explain the reduction mechanism of the sulphydryl group. The three factors, diffusion of hydrogen, the metal electrode, and the nature of the sulphydryl group will be discussed.

Diffusion of hydrogen.

Dixon bases the assumption that hydrogen diffuses away from the platinum electrode on three sets of experimental results: (1) there is a drift in the potential of a platinum electrode in a solution of cysteine; (2) although cysteine does not give a reduction potential with the platinum electrode sufficiently high to indicate its power to reduce indigo carmine, Dixon found that indigo carmine is reduced by cysteine; (3) Wieland showed that finely divided platinum or palladium will act as a hydrogen acceptor with certain hydrogen donators, such as quinol, indigo white and hydrazobenzene, that hydrogen will be absorbed by the platinum, and that the oxidant form of these reversible compounds may be shown as a product of the reaction in the solution.

Drift in reduction potential.

Dixon and Quastel [1923] first observed the drift in the reduction potentials of a solution of cysteine. Kendall and Nord [1926] confirmed this observation and we will record a more detailed study of this phenomenon. The platinum electrodes used in the work of Kendall and Nord and in this investigation were placed, as a routine, in boiling concentrated nitric acid shortly before they were used; the acid was then thoroughly washed off with tap water and then with distilled water and the electrodes were placed in phosphate buffer, p_H 7·4, solution in the electrode cells and allowed to remain for from 60 to 90 minutes during which time oxygen-free nitrogen was passed through the solution. 6 cc. of 0·1 *N* cysteine hydrochloride solution, oxygen-free, was then added to the 100 cc. of phosphate buffer, which contained sufficient sodium hydroxide to neutralise the hydrochloric acid present in the cysteine solution. The potential was then read during the following 6 to 8 hours and it was found that a slow drift in the potential continued until the maximal potential was reached which was not greater than − 0·14 v. However, it was noticed that on some days the reduction potential of cysteine recorded on the platinum electrode was much higher than the value just mentioned and, moreover, that the potential approached the equilibrium point much more rapidly. After this had been observed several times it was shown that the electrodes which behaved in this manner had stood exposed to air for a much longer time than usual. On the last occasion when these high readings were obtained it was noted that the electrodes had been exposed to air for 75 hours after treatment

with nitric acid. In this connection Dixon and Quastel stated that the reduction potential "was determined by previous history of the platinum electrode used." It appeared probable that the previous history of the platinum electrode was largely a result of the influence of nitric acid and oxygen. In order to determine to what extent oxygen or hydrogen on the platinum electrode affected the drift in the potential, a polarising current was used of such strength that hydrogen in minute bubbles was evolved from the platinum electrode. The oxygen was liberated on the surface of a second platinum electrode, surrounded by a glass tube, which permitted the immediate and almost quantitative removal of oxygen gas from the solution. A rapid flow of oxygen-free nitrogen removed oxygen from the space above the liquid. With platinum electrodes treated in this manner reduction potentials of cysteine (added to the buffer) less than − 0·14 v. were never observed. If the reduction potential was determined immediately after polarisation it was, of course, high, owing to adsorbed hydrogen, but this value slowly decreased until the value − 0·14 v. was reached below which it did not drop. Variations in the actual potential are discussed further on.

In order to show the effect of oxygen adsorbed on the surface of the platinum, anodic polarisation of the electrode in a phosphate buffer, p_H 7·4, was carried out for 30 seconds, resulting in liberation of minute bubbles of oxygen on the surface of the platinum electrode. The hydrogen liberated from the second platinum electrode was removed through a glass tube which surrounded the platinum cathode. After polarisation, as would be expected, the platinum electrode indicated a high oxidation potential; however, the significant observation was then made that a very long time was required after the addition of 6 cc. of 0·1 *N* cysteine for the reduction potential to reach the value − 0·14 v.

Although platinum is recognised as one of the most efficient surfaces to catalyse oxidation processes, the fact remains that the oxygen adsorbed on the surface of the platinum delayed the development of the reduction potential to its former value for 2 or more hours.

The slow increase in the reduction potential of a solution of cysteine after anodic polarisation is in striking contrast to the almost instantaneous increase which follows the addition of 0·5 cc. of 0·02 *N* reduced indigo or reduced indigo carmine to a phosphate buffer, p_H 7·4, in which is suspended a platinum electrode which has been treated with anodic polarisation. There is immediate interaction resulting in oxidation of the reductant with the minute amount of oxygen on the platinum and then the excess of the reductant registers the expected reduction potential. In the case of reduced indigo the platinum is found covered with a fine film of indigo.

If anodic polarisation is carried out in a solution of cysteine an oxidising potential of + 0·24 v. is produced on the surface of the platinum which is followed by a slow drift to the value − 0·14 v. If to such a solution 1 cc. of 0·02 *N* reduced indigo or reduced indigo carmine is added the potential almost

immediately increases to that expected from the reduced indigo or reduced indigo carmine.

These results show that instead of the sulphydryl group giving off hydrogen so easily that it is a representative of a class of substances which differ from all other reductants because the reaction $AH_2 \rightarrow A + HH$ is irreversible, it would appear that the reverse of this explanation is the correct one, and that hydrogen dissociates from reduced indigo carmine or reduced indigo far more readily than it does from the sulphydryl group. Dixon assumes that the drift in the potential is because dissociation of hydrogen from the R.C.SH group and diffusion of hydrogen from the electrode have not reached a "steady state." These results indicate that the drift is due to the slow interaction between the sulphydryl group and oxygen on the surface of the platinum.

The reduction of indigo carmine by cysteine.

From an investigation of the reduction potential of solutions of cysteine Dixon concluded that the reduction intensity expressed on the r_H scale was 14, and since this intensity of reduction is insufficient to reduce indigo carmine, which has an r_H value of 8, it was indicated that cysteine could not reduce indigo carmine. However, Dixon found that cysteine did reduce indigo carmine, and was therefore forced to find some compensating process which prevented the cysteine from exerting its actual reduction intensity on the metal electrode. It appeared to Dixon that the most probable compensating mechanism was the diffusion of hydrogen away from the platinum electrode.

It is evident, however, that if the observation concerning the ability of cysteine to reduce indigo carmine was in error then no compensating process preventing the cysteine from exerting its true reduction intensity is needed. The results of Kendall and Nord [1926], and still further evidence in this paper, show that in the absence of an activating agent cysteine cannot reduce indigo carmine. The value of the reduction intensity of cysteine in the absence of an activating agent given by Dixon and Quastel has been confirmed and we may conclude that the reduction intensity of a solution of cysteine reaches the limiting value of about – 0·14 v. A substance with this reduction potential cannot reduce indigo carmine and this has been demonstrated experimentally.

This type of reasoning, however, may lead to erroneous conclusions. Kendall and Witzmann [1927] have shown that adrenalone has almost no effect on the platinum electrode but it can reduce indigo carmine. Kendall and Ort [1926] have shown that 2-oxodihydroindole-3-propionic acid can reduce dibromophenolindophenol although it has no effect on the platinum electrode.

Adsorption of hydrogen from a reductant with platinum.

In support of the theory of the "steady state" Dixon cited the observations of Wieland [1912, 1913] that platinum would adsorb hydrogen dissociating from quinol, and that quinone could be shown to be present in

solution. He assumes that a similar reaction is taking place in solutions of cysteine resulting in accumulation of hydrogen on the surface of the platinum and the formation of cystine in solution. It is possible that the reaction suggested by Dixon could occur in solutions of cysteine provided the platinum were highly activated and sufficient platinum were present to act as a hydrogen acceptor to an extent great enough to influence the reaction. However, it can be readily shown that with the platinum electrode used in this work the small surface of the platinum is not sufficient to bring about such a reaction even with substances far more reactive as hydrogen donators than is cysteine. For example, 1 cc. of 0·004 *N* reduced indigo or of reduced indigo carmine is stable in the presence of the platinum electrode in 100 cc. of phosphate buffer, p_H 7·4, for many hours. There is no change in the colour of the solution and the reduction potential slowly increases to a constant value after 2 or 3 hours. If hydrogen were removed from these reductants by the platinum the solution would become blue and the potential would decrease as the reaction proceeded. If diffusion of hydrogen occurs at all it is to an extent totally insufficient to determine the reduction potential.

If then the platinum electrode used cannot function in this manner with so easily oxidised a compound as reduced indigo, it becomes utterly impossible to explain the reduction potential of a substance such as cysteine by assuming that hydrogen is dissociating from the sulphydryl group and is then diffusing from the platinum surface.

If this reaction cannot be shown to occur with the platinum electrode the probability of its occurring with the gold electrode may be entirely dismissed.

Different metal electrodes.

Dixon and Quastel observed continuous and extensive drifts in the reduction potential of cysteine solutions when platinum was used; they observed smaller fluctuations in the potential when a gold electrode was used. Without actually explaining the reason for the drift Dixon [1927] assumed that the electrode functioned as a way of escape for hydrogen dissociating from the sulphydryl group and that the speed with which 2H was converted into H_2 on the surface of the metal actually determined the reduction potential of the solution.

The results in the preceding section concerning the influence of traces of hydrogen or oxygen on the platinum electrode appeared to explain the drift in a solution of cysteine when platinum was used. We then carried out similar experiments with gold electrodes. By immersing a gold and platinum electrode in the same solution of cysteine the influence of traces of oxygen and hydrogen on the surface of these two metals was shown. After cathodic polarisation in a solution of 6 cc. of 0·1 *N* cysteine in 100 cc. of phosphate buffer, p_H 7·4, the gold electrode resumed its former potential with a little greater speed than did the platinum, but after anodic polarisation for from

30 to 60 seconds the gold promptly gave up all traces of oxygen and the potential previous to the polarisation was rapidly attained. We consider, therefore, that this result is the correct explanation for the difference in the behaviour of gold and platinum electrodes, and it consists merely in a difference in the speed with which the sulphydryl group can react with oxygen adsorbed on the surface of the platinum or the gold. This is probably equivalent to a measure of the amount of oxygen on the metal surfaces.

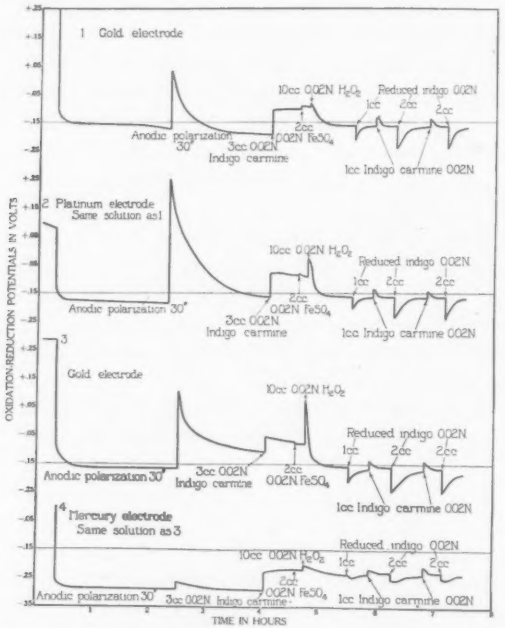

Fig. 1. The reduction potential of a solution of cysteine on gold, platinum and mercury electrodes.

The gold and platinum electrodes used to obtain Curves 1 and 2 were placed in the same solution of 100 cc. of phosphate buffer, p_H 7·4, containing 6 cc. of 0·1 *N* cysteine. The gold and mercury electrodes shown in Curves 3 and 4 were placed in the same solution, which was identical with the solution represented in Curves 1 and 2. The platinum electrode (Curve 2) was treated with cathodic polarisation in phosphate buffer just before it was placed in the solution of cysteine. The gold electrodes were not treated with cathodic polarisation. Oxygen liberated on the surface of the gold and platinum electrode (Curves 1 and 2) caused a marked decrease in reduction potential. The gold electrode returned to its former value much more rapidly than did the platinum electrode. Anodic polarisation of the gold and mercury electrodes, Curves 3 and 4, produced a decrease in reduction potential on the gold electrode and almost no effect on the mercury electrode. The addition of indigo carmine at 5 hours and 35 minutes on Curves 1 and 2 produced a drop to approximately the same potential on the gold and platinum electrodes. The addition of the same volume of indigo carmine to the solution, Curves 3 and 4, produced a slight decrease on the mercury, but the reduction potential was almost 200 millivolts higher than on the gold. Addition of 2 cc. of 0·02 *N* ferrous sulphate to each solution produced but a trifling change. The addition of 10 cc. of 0·02 *N* hydrogen peroxide which resulted in formation of the third component brought the equilibrium point of both solutions to almost the same potential on the two gold electrodes and the platinum electrode. The corresponding potential on the mercury electrode was much higher. Addition of 0·02 *N* reduced indigo in 1 and 2 cc. portions produced effects on the platinum and gold electrodes which are almost identical and the final equilibrium point on all three electrodes is identical. The difference in potential between the mercury and the gold and platinum electrodes is assumed to be due to the difference in the quantitative influence of the oxidant, R.C.S form of cysteine.

In the same way the effect of hydrogen and oxygen on the mercury electrode was determined by immersing mercury and platinum, mercury and gold or all three metal electrodes in the same solution of cysteine. Dixon regards the difference in the potential registered by the gold and mercury electrodes when both are immersed in a solution of cysteine as good evidence in support of the assumption of a "steady state," and concludes that hydrogen dissociating from the sulphydryl group produces a much higher reduction potential on the mercury electrode than on the gold; a result which would be expected from the high over-voltage of hydrogen on the mercury electrode. Dixon found that the mercury electrode was 200 millivolts negative to a gold electrode immersed in the same solution. We have repeated this experiment and have confirmed a potential difference which depends upon the condition of the metal surfaces. We have interpreted the observation in a very different manner.

Before giving an explanation of this potential difference we shall refer to some facts which have been established in the general field of oxidation-reduction phenomena. Dixon points out that hydrogen is available to the platinum electrode from three sources, one of which is the reductant, AH_2. In a solution of cysteine he assumes that the R.C.SH grouping is the reductant and that the R.C.SS.C.R grouping has no effect on the potential. The entire mechanism of the reduction potential must involve the R.C.SH group alone.

Through some work which has recently been completed in this laboratory evidence has been secured showing that there is a great difference between the chemical reactivity of the R.C.SS.C.R grouping and that of the R.C.S portion of the R.C.SH grouping.

It has been conclusively shown that the reductant, R.C.SH, is not immediately oxidised to R.C.SS.C.R, which is without effect upon the platinum, but that the oxidant, R.C.S, or its chemical equivalent, exists in a definite concentration and is capable of affecting the metal electrode. In fact the R.C.S or oxidant form of cysteine is the determining factor of the reduction potential of cysteine; and when this assumption is made there is no necessity for a kinetic equilibrium between dissociation of hydrogen from the reductant and diffusion of hydrogen from the metal electrode.

The equilibrium of reductant and oxidant will be discussed with more detail, but for the present it only becomes necessary to assume that R.C.S or its chemical equivalent is the oxidant form of cysteine in order to explain the difference in the potential of the gold and mercury electrodes. Clark has shown that the oxidation-reduction potential is equal to a function of the logarithm of the ratio, reductant/oxidant, and it can be assumed either that a certain concentration of electrons is established in the solution by this ratio or that both electrons and the oxidant form of the compound affect the metal, the electrons by increasing the concentration of the electrons on the surface of the metal and the oxidant by removing electrons from the surface of the metal. With certain substances there is no difference in the reduction potential

of a solution containing oxidant and reductant forms when measured on different metal electrodes. Dixon has shown that a solution containing quinhydrone has the same potential on mercury and gold; however, it can be shown that this is not always true and that the oxidant of a compound may influence one metal more than another.

We shall now suggest an explanation for the fact that the reduction potential indicated by the mercury was approximately 0·2 v. higher than that given by either the platinum or the gold. The increased reduction potential indicated on the mercury may readily be due to the difference in the effect of the oxidant, R.C.S, on the mercury and on the gold or platinum. Two groups of experiments confirmed this assumption. The first is that anodic polarisation of the mercury electrode or the addition of hydrogen peroxide does not result in a significant drop in the reduction potential of a cysteine solution. Anodic polarisation of platinum produces a great decrease in the reduction potential. The same treatment of a gold electrode produces a marked but less effect. We can, therefore, conclude that in a solution of cysteine the effect of oxygen on mercury is much less than it is on gold or platinum. This experimental fact justifies the assumption that the oxidant, R.C.S, may have but slight effect on the mercury electrode. If this is true, cysteine would produce a greater apparent reduction potential on this metal, but even with the mercury electrode the potential is determined by an equilibrium between R.C.SH and R.C.S and not by a kinetic equilibrium between dissociation and diffusion of hydrogen. This is conclusively shown by the failure of cysteine to reduce indigo carmine even in the presence of mercury. If the sulphydryl grouping in the presence of mercury were actually dissociating into hydrogen with the formation of cystine, then certainly this hydrogen would be available for the reduction of indigo carmine, but the addition of 0·5 cc. of 0·02 *N* indigo carmine to a solution of fifty times the equivalent amount of cysteine in phosphate buffer, p_H 7·4, in an oxygen-free electrode cell containing 5 cc. of mercury on the bottom of the electrode chamber, showed that this small amount of indigo carmine was stable in the presence of this enormously larger amount of cysteine.

Still more convincing proof, however, was obtained by placing all three, gold, platinum and mercury electrodes, in 100 cc. phosphate buffer, p_H 7·4, which was entirely free from oxygen. To this solution 3 cc. of 0·02 *N* indigo carmine and 3 cc. of 0·02 *N* reduced indigo carmine were added. It was then observed that, in agreement with the effect of quinhydrone on gold and mercury electrodes reported by Dixon, this equal mixture of the oxidant and reductant forms of indigo carmine produced precisely the same reduction potential with gold, platinum and mercury electrodes. It is, therefore, evident that the oxidant form of indigo carmine can affect gold, platinum and mercury to the same degree. To this solution 2 cc. of 0·1 *N* neutral cysteine was added. There was no change in the potential indicated by the platinum and gold electrodes, but the reduction potential of the mercury electrode increased by

0·052 v. If hydrogen from the sulphydryl group was diffusing from the mercury it should be available to interact with the indigo carmine and reduce the dye until equilibrium was again established. This, however, did not occur. This result is entirely in keeping with the hypothesis that on each metal the potential is determined by the ratio R.C.SH/R.C.S, and that the effect of both R.C.SH and R.C.S varies with the metal of the electrode. The reduction potential is apparently increased on the mercury only because of the small effect of the oxidant, R.C.S.

Since the three metals, gold, platinum and mercury, showed the same oxidation-reduction potential in the presence of quinhydrone and indigo carmine, experiments were made to see whether these metals indicated a difference in the reduction potential when other dyes were used. The three

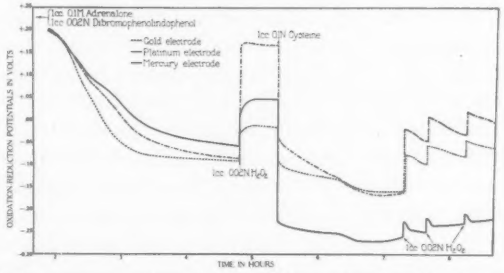

Fig. 2. The oxidation-reduction potential of a solution containing adrenalone, dibromophenol-indophenol, hydrogen peroxide and cysteine on gold, platinum and mercury electrodes.

1 cc. of 0·1 *M* adrenalone hydrochloride was added to 100 cc. phosphate buffer, p_H 7·4, in an electrode cell free from nitrogen. 1 cc. of 0·02 *N* dibromophenolindophenol was added at 1 hour and 38 minutes. The chart shows the oxidation-reduction potential on the three metal electrodes during the following 6 hours. 1 cc. of 0·02 *N* hydrogen peroxide was added at 4 hours and 48 minutes, at 7 hours and 18 minutes, at 7 hours and 38 minutes, and at 8 hours and 13 minutes. 1 cc. of 0·1 *N* cysteine was added at 5 hours and 21 minutes. The presence of cysteine reverses the effect of hydrogen peroxide on the mercury and gold electrodes.

electrodes were placed in a solution of 100 cc. of phosphate buffer, p_H 7·4, containing 1 cc. of 0·1 *M* adrenalone (Fig. 2). 1 cc. of 0·02 *N* dibromophenol-indophenol was then added. As long as both oxidant and reductant forms of the dye were present the potential on all three electrodes agreed within a fraction of a millivolt. As the last traces of the oxidant form of the dye disappeared the reduction potential indicated on the gold increased rapidly, assuming a steady potential after about 3 hours. The reduction potential of the same solution indicated on the platinum electrode increased slowly and at the end of almost 3 hours it was very close to the potential indicated on the gold. The reduction potential shown on the mercury electrode increased much more slowly, but at the end of almost 3 hours it was still increasing at a rate which indicated that after a long time it might reach the potential shown by the gold and the platinum. This part of the curve is therefore significant in showing the velocity with which the substances in solution come into equilibrium with the metal. At the time indicated by 4 hours

and 48 minutes (Fig. 2) 1 cc. of 0·02 *N* hydrogen peroxide was added. This caused a drop of potential on the gold electrode of less than 80 millivolts. The mercury electrode was affected much more and the platinum electrode indicated a drop in reduction potential of more than 250 millivolts. At the time indicated by 5 hours and 21 minutes, 1 cc. of 0·1 *N* cysteine was added. This produced a remarkable effect in which the reduction potential on the electrodes was reversed. The platinum still remained lowest, the gold was but little higher than the platinum but there was a marked and immediate rise in the reduction potential on the mercury electrode. The potential indicated on the three electrodes showed slow drifts, and at 7 hours and 18 minutes the gold and platinum electrodes were again approximately the same and the mercury was negative to both the gold and platinum by a difference of 100 millivolts. 1 cc. of 0·02 *N* hydrogen peroxide was added. This produced a marked drop in the platinum, a slight drop in the gold and almost no effect on the mercury electrode. The same amount of hydrogen peroxide was again added at 7 hours and 39 minutes and at 8 hours and 14 minutes.

The reaction of the metal electrodes to hydrogen peroxide depends upon the substances in solution. In the presence of adrenalone, reduced dibromophenolindophenol and a small amount of the oxidation product of adrenalone, hydrogen peroxide affects the mercury electrode more than it does the gold electrode; however, in a solution of cysteine the reverse is true. These results are sufficiently quantitative and significant to indicate the nature of the difference in potential between the gold and mercury electrodes in a solution of cysteine and it cannot be assumed, merely because there is a difference, that hydrogen is dissociating from the sulphydryl group and diffusing from the surface of the metal electrodes.

The nature of the sulphydryl group.

Dixon and Quastel [1923], by changing the ratio of cysteine to cystine, found that variations in the reduction potential were produced, but the results did not agree with the curve plotted from the usual equation to express oxidation-reduction potentials. It became apparent that the reduction potential was a function of the logarithm of the concentration of cysteine, and that the concentration of cystine had no effect on the potential. In discussing the possible reactions involved Dixon and Quastel state, "If the (R.SS.R) term is to be regarded as a constant, it is obvious that this cannot represent the concentration of cystine. It seems almost necessary, therefore, to postulate the possible presence of an intermediate compound which would be the 'true' oxidised form of cysteine and is transformed irreversibly into cystine. In such a case the concentration of this intermediate oxidised substance would appear to be maintained at a constant value—as though the solution in presence of cysteine (or glutathione) must be 'saturated' with respect to this particular compound." This viewpoint, however, was abandoned by Dixon, who published a paper [1927] assuming that the dissociation of hydrogen

from cysteine resulted in the immediate formation of cystine and that this reaction is irreversible, within the limits of the experiment.

We have submitted evidence in this paper to show that hydrogen does not dissociate from the sulphydryl group, and there are strong grounds for assuming that the R.C.S radical does not immediately form cystine but that it exists in a definite although limited concentration. A new method for the determination of oxidised and reduced glutathione or of cystine and cysteine, based on the difference in chemical activity of the R.C.SS.C.R and R.C.S groups, will be published from this laboratory. If the assumption is made that the concentration of R.C.S is a constant the curve relating reduction potential to concentration of cysteine becomes logarithmic in nature. This has been found to be true by Dixon and Quastel, and we have confirmed their results.

By assuming the kinetic equilibrium $[R.C.SH]^2 = K\,[H]^2$, relating dissociation of hydrogen to the diffusion of hydrogen, Dixon was able to show that the same equation was obtained. This, however, is not proof that a kinetic equilibrium does exist. Finally, it may be said that cysteine reacts as a substance which exists in three forms. A reductant, R.C.SH, which produces a reduction potential on a metal electrode, which has a definite limiting value; this is about $-0{\cdot}14$ v. When cysteine is oxidised the oxidant accumulates in the solution up to a certain limit and then passes into a form which does not affect the platinum electrode; however, the oxidant form, R.C.S, reacts as any other oxidant in contact with a metal electrode and limits the reduction potential of the solution of cysteine.

The reduction potentials which have been given throughout this paper for a solution of cysteine have been stated as definite, unqualified values. Observation of the reduction potentials of various solutions, however, does not agree with the assumption that the values are definite or have any particular significance. All of the solutions considered have been "unactivated."

In solutions of cysteine and cystine there is no known way of increasing the concentration of the oxidant R.C.S: the potential is a function of the logarithm of the concentration of cysteine; however, it has been shown by Kendall and Nord [1926] that in the presence of indigo carmine, hydrogen peroxide and sodium disulphide can activate cysteine through the formation of an unknown third component. In the presence of this component the reduction potential is not established by the concentration of cysteine only, but is in proportion to the ratio of the —SH to —SS groups. Since this can be shown to be true in solutions activated in this manner it would be remarkable if a similar condition were not present in some solutions of cysteine not activated by indigo carmine and hydrogen peroxide, and during this investigation there have been many observations which indicated the presence of "activated" solutions of cysteine and cystine. The activating mechanism is unknown, but the extraordinary sensitiveness of the solution of cysteine to minute traces of impurities has become apparent during the investigations

of this series of compounds. Paradoxes and surprising inconsistencies in the reduction of dyes and in the reduction potentials are frequently met with which cannot be explained, but which are accounted for if it is assumed that the presence of some activating agent is essential before the —SH and —SS groupings can manifest their respective maximal activities.

While the intensity of reduction and oxidation can be explained only by activation of the —SH and —SS groups, drifts in reduction potential of unactivated solutions may be due to other causes. Dixon and Quastel state that there is a normal drift of the platinum electrode toward more positive readings, and that in the presence of cysteine the potential due to cysteine is fairly rapidly reached and that this then slowly falls in the usual manner of non-cysteine solutions. We cannot confirm this observation. When the reduction potential of a solution of cysteine has reached a constant value it does not fall, but remains practically uniform for many hours (Fig. 1). The extreme sensitiveness of the platinum electrode to traces of oxygen in such a solution suggests that in the experiment of Dixon and Quastel the nitrogen used contained traces of oxygen.

—SH/—SS RATIO IN ACTIVATED SOLUTIONS.

If a small amount of indigo carmine (0·5 cc. of 0·02 *N* solution) is added to a solution of cysteine which is not activated, the reduction potential drops to a value of about − 0·14 v., irrespective of its previous value. This is evidence that the solution contains no poising compound, and that the reduction potential was not determined by the ratio of total reductant to total oxidant.

If a solution containing equal amounts of cysteine and cystine and the third component is prepared as described in the previous paper [Kendall and Loewen, 1928, p. 668], in seven electrode cells, the solutions will all be of the same colour and the reduction potential of all of them will agree within 3 millivolts. If to six cells, 1, 2, 3, 4, 5 and 6 cc., respectively, of 0·1 *N* cystine (as the sodium salt) and an equivalent amount of 0·1 *N* sulphuric acid are added, there is no change in the p_H but the potentials of the solutions vary in agreement with the amount of cystine added. Still more significant, the velocity of oxidation of reduced indigo is found to depend on the ratio of the —SH to the —SS grouping. The reduction potentials and the velocity of oxidation of these solutions are shown in Fig. 3. Since the reduction intensity of a solution containing cysteine and the third component was lowered by the addition of cystine it follows that the concentration of the oxidant was increased. Assuming the oxidant to be R.C.S it is evident that the ultimate effect of the third component is to act on cystine in such a manner that the stable —SS linkage becomes weakened to such an extent that the concentration of the R.C.S radical is increased.

Although no evidence has been submitted by Dixon and Tunnicliffe [1927] which disproves the hypothesis that the third component is an oxygen or sulphur

addition product of cysteine, on the other hand no convincing evidence can be submitted that the third component has this exact structure; but it was pointed out that the significant influence on these —SH and —SS groups was the ease with which the sulphur atom could change its valence, and the work which is reported in this communication further emphasises the importance of this observation. The R.C.SH and R.C.SS.C.R groupings are relatively stable, but in simple phosphate buffer solution they can be activated.

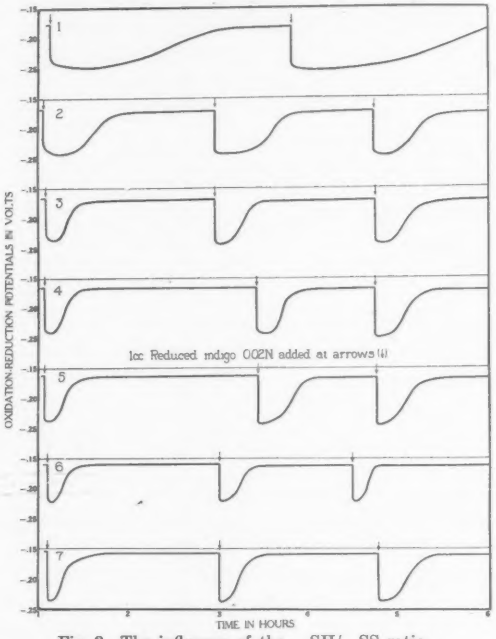

Fig. 3. The influence of the —SH/—SS ratio.

The velocity of oxidation of reduced indigo in seven solutions, each containing 1·5 cc. of 0·1 *N* cysteine and 1·5, 2·5, 3·5, 4·5, 5·5, 6·5, 7·5 cc. of 0·1 *N* cystine, respectively. The same volume (30 cc.) of a solution containing the third component activating the cysteine and cystine was present in each solution. Both the equilibrium potential and the velocity of oxidation of reduced indigo in each solution are determined by the ratio of the —SH to the —SS groupings.

The statement of Dixon and Tunnicliffe that the results reported by Kendall and Nord have but limited application since they refer only to the particular dye, indigo carmine, and do not occur with other dyes, such as methylene blue, shows that they have not taken cognisance of the phenomena involved. Whether the sulphydryl group dissociates with the liberation of hydrogen, or whether it is a relatively stable grouping and can function only after activation by a second compound may appear to be two hypotheses of only academic significance, but the problem concerning the relation of these groups to biological oxidation is still unanswered. The hypothesis suggested

by Kendall and Nord that the —SH and —SS groups can be and are activated has not been shown to be incorrect. The assumption that hydrogen is dissociating from the sulphydryl group cannot be accepted even as a working hypothesis.

SUMMARY.

Dixon's hypothesis of a "steady state" depending on a kinetic equilibrium between the dissociation of hydrogen from cysteine and diffusion of hydrogen from the surface of the metal electrode, has been shown to be incorrect. The drift in the reduction potential with a platinum electrode has been shown to be due to traces of oxygen, or other oxidising agents, adsorbed on the surface of the metal. The steady potential given with a gold electrode is explained by the failure of the gold to adsorb oxygen. The higher reduction potential with the mercury electrode has been shown to be due to failure of the oxidant of the system to affect the mercury to as great an extent as it does platinum and gold. The difference in potential between the gold and mercury electrodes in a solution of cysteine cannot be explained by the higher over-voltage of hydrogen on mercury.

When cysteine is oxidised it does not immediately form cystine, but an intermediate compound exists which is the oxidant of the system. The oxidant of the system appears to exist in a low and limited concentration above which it passes into cystine. In the absence of an activating agent, cystine does not affect the platinum electrode and the reduction potential is a function of the logarithm of the concentration of cysteine. In a solution of cysteine and cystine, containing an activating agent, cystine is brought into equilibrium with the oxidant of the system and the reduction potential is determined by the ratio C.SH/C.SS.C groups.

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LXXXIII. THE REDUCTION POTENTIAL OF CYSTEINE.

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It has been shown by Dixon and Quastel [1923] that the reduction potential of cysteine-cystine mixtures depends entirely on the concentration of the reduced form, cysteine. Cystine seems to exert no potential under the experimental conditions employed. The suggestion made to explain this anomalous behaviour was contained in the following words: "It seems almost necessary, therefore, to postulate the possible presence of an intermediate compound which would be the "true" oxidised form of cysteine and which is transformed irreversibly into cystine. In such a case the concentration of this intermediate oxidised substance would appear to be maintained at a constant value, as though the solution in presence of cysteine must be "saturated" with respect to this particular compound."

Dixon [1927] has advanced another interpretation of this phenomenon based upon an extension of the Wieland theory of activation of hydrogen. According to Dixon, when an electrode is placed in a solution containing a substance AH_2 which gives a reduction potential, the electrode surface becomes "more or less completely covered with hydrogen atoms loosely attached to the surface." This hydrogen "which corresponds to the 'active hydrogen' of Wieland must not be regarded as atomic hydrogen since it is in combination with the platinum. The attachment is, however, only loose so that it does actually approximate to atomic hydrogen in behaviour." The hydrogen in this state is in equilibrium with hydrogen molecules, with AH_2 molecules and with hydrogen ions, and the potential is determined by the pressure of hydrogen at the electrode and the concentration of hydrogen ions. Now in interpreting the potential of cysteine-cystine mixtures on this view it is necessary to conclude "that hydrogen atoms can be transferred from the sulphydryl group to the electrode, but that the disulphide form is quite incapable of taking hydrogen back again from the electrode," the system being essentially an irreversible one. But, as Dixon points out, if the hydrogen is transferred irreversibly to the electrode it is to be expected that the pressure of hydrogen will rise to high values, the rate at which the rise occurs being dependent upon the rate of transference of hydrogen from the cysteine to the

electrode. Thus the reduction potential should become more and more negative. Experiment has shown, however, that a steady potential is produced by a given quantity of cysteine, and to explain this Dixon has sought a compensating process, which he has found in the diffusion of hydrogen gas from the electrode. The potential of cysteine is secured, he maintains, by the attainment of a steady state at the electrode which occurs when the velocity of transference of hydrogen atoms to the electrode from the cysteine is balanced by the velocity of diffusion of hydrogen gas from the electrode.

The two processes are represented by the equations:

$$2\text{R.SH} \rightarrow \text{R.SS.R} + 2\text{H}, \qquad \ldots(1)$$

$$2\text{H} \rightarrow \text{H}_2. \qquad \ldots(2)$$

The velocity v_1 of reaction (1) is $K_1\,[\text{R.SH}]^2$; the velocity v_2 of reaction (2) is $K_2\,[\text{H}]^2$ where K_1 and K_2 are constants. The steady state occurs when $v_1 = v_2$.

So that $$K_2\,[\text{H}]^2 = K_1\,[\text{R.SH}]^2$$

or $$[\text{H}] = \sqrt{\frac{K_1}{K_2}}.[\text{R.SH}].$$

The value of [H], *i.e.* of K_1, K_2 and [R.SH], determines the potential secured.

Therefore, it is clear that if the velocity constants K_1 or K_2 can be altered, there will be a change in the steady state at the electrode and hence in the magnitude of the reduction potential. The H in equations (1) and (2) corresponds, as Dixon says, to the "active hydrogen" of Wieland. It is the hydrogen which is taken up by "acceptors" such as quinone or methylene blue. The reduction of methylene blue by cysteine, on the Wieland view, must be regarded as due to the transference of active hydrogen from one molecule to the other, the hydrogen in the *reactive* cysteine molecules being considered in an active condition. Now the rate of reduction must be dependent upon the rate of formation of *reactive* cysteine molecules and therefore it is dependent upon the rate of formation of "active hydrogen."

If, then, keeping the concentrations of cysteine and methylene blue constant, the rate of reduction is increased by the addition of a catalyst, it must be concluded that the effect of the catalyst is, virtually, to increase the rate of formation of active hydrogen from the cysteine (*i.e.* to increase the number of fruitful collisions by increasing the lability of the hydrogen of the sulphydryl group). The effect of a catalyst, therefore, is to increase the velocity constant K_1 of equation (1) to a new constant K_3.

Let us now consider an electrode present in a solution of cysteine. A steady state is reached when

$$[\text{H}] = [\text{R.SH}]\sqrt{\frac{K_1}{K_2}}.$$

Let a small quantity of a catalyst, *e.g.* a trace of cupric or ferric ion be added

to the solution. Then since K_1 is increased to K_3, a new steady rate will be reached given by

$$[H] = [R.SH] \sqrt{\frac{K_3}{K_2}}.$$

It cannot be maintained, for the purposes of this argument, that the electrode has properties so essentially different from methylene blue that whereas the effect of an added catalyst occurs in the latter case it does not in the former; for if the effect of a catalyst (assumed to be specific for the hydrogen donator) is to render more labile the bonds between the hydrogen atoms and the rest of the molecule, with resulting increased fruitful collisions with a hydrogen acceptor (*i.e.* an increased rate of transference of active hydrogen), such an increase in fruitful collisions must be observed with any hydrogen acceptor including the electrode; for this, according to Dixon, must be a hydrogen acceptor, or otherwise a potential would not be secured.

In the change of steady state from $[R.SH] \sqrt{\frac{K_1}{K_2}}$ to $[R.SH] \sqrt{\frac{K_3}{K_2}}$ induced by the catalyst the assumption has been made that K_2 is unaltered. This appears to us a reasonable assumption as there is no evidence to suppose that the catalyst in question can affect the velocity of diffusion of hydrogen gas from the electrode.

Hence it is to be expected on Dixon's view that the (hypothetical) steady state at the electrode should be changed on the addition of a catalyst with a resulting increase in the negative reduction potential.

Experiments described in this paper show that purified metal-free cysteine, which reduces methylene blue very slowly, gives a high negative reduction potential in presence of an electrode which is itself not catalytically active. This potential is *not* increased by the addition of a catalyst ($Cu^{\cdot\cdot}$ or $Fe^{\cdot\cdot\cdot}$) although under the same conditions the rate of reduction of methylene blue is increased tenfold.

It would seem, therefore, that Dixon's view of the mechanism by which the potential of the sulphydryl group is obtained at the electrode, is untenable. It seems more likely that the interpretation already suggested (Dixon and Quastel) is correct. Let us suppose that the hypothetical intermediate substance which is the "true" oxidised form of R.SH is the radical R.S and that this is transformed irreversibly into R.SS.R. Then the equations which account for the facts would be as follows:

$$\begin{aligned} R.SH &\rightleftharpoons R.S^- + H^+ && \text{reversible} \\ R.S^- &\rightleftharpoons R.S + \ominus && \text{reversible} \\ 2R.S &\rightarrow R.SS.R && \text{irreversible} \end{aligned}$$

the presence of a catalyst being without effect on the equilibrium point of the system, and hence on the reduction potential observed. We have, however, no definite evidence yet for the existence of R.S, our suggestion of its presence being purely tentative.

Experimental.

Reduction of methylene blue by metal-free cysteine in presence of the gold electrode.

Since it was desirable that an electrode should be used which was free from catalytic action on the reduction of methylene blue, preliminary tests were made to determine whether the electrode used in our experiments was inert in this respect. Experiments showed that the rate of reduction of methylene blue by metal-free cysteine was not appreciably affected by the presence of the gold electrode. The metal-free cysteine was prepared by the method of Harrison [1924] and the reduction was carried out by the technique described by him [1927]. The pure gold electrode was a plate 1 cm. by 2·5 cm. and 0·5 cm. thick, joined to a pure gold wire. Reductions were carried out *in vacuo* at p_H 7·4 (phosphate buffer) and 37°.

Reduction potentials of cysteine in presence of the gold electrode.

The electrode vessel consisted of a closed tube of Pyrex glass, through which a stream of pure nitrogen was passed by means of a tube dipping in the solution. The nitrogen was purified by passing it through a series of five alkaline pyrogallol solutions and two solutions of alkaline sodium hydrosulphite and finally through a dilute solution of sodium hydroxide. The bubbling was continued while observations were taken, this being quite an efficient method of stirring. A calomel cell (containing saturated potassium chloride solution) was connected by means of an agar-salt bridge to the solution in the vessel.

In taking readings the procedure was to pipette a definite volume of a phosphate buffer solution (p_H 7·4) into the electrode vessel (thoroughly cleaned with acids), and to bubble nitrogen through until the gold-calomel potential had reached a steady value. The gold electrode was washed by immersing it a number of times in boiling water, after an initial treatment with hot acids. After this washing the electrode gave readings which varied between + 0·07 v. and + 0·09 v. If such an initial value were not obtained, the electrode was re-washed and the contents of the vessel rejected. A known volume of the pure cysteine solution was then introduced into the vessel and the potential of the solution, when steady, was determined by the potentiometer in the usual way. The temperature of the electrode vessel and calomel cell was maintained constant throughout each experiment by immersion in a thermostat.

The metal-free cysteine solution, which was prepared freshly in each experiment with quartz-distilled water and made up to the correct p_H with pure sodium hydroxide, was kept in a well evacuated tube. All tubes containing solutions used in the experiments were evacuated, filled with nitrogen and re-evacuated.

The following is a typical experiment. In the electrode vessel were placed 17·2 cc. of quartz-distilled water and 2·5 cc. of phosphate buffer (Clark and Lubs), p_H 7·4. Nitrogen was run through the solution and the steady potential obtained was + 0·072 v., the temperature during the experiment being 19°. 0·3 cc. of cysteine solution (containing 2·42 mg. pure cysteine) was then added to the electrode vessel, making the final concentration of cysteine $N/1000$. The potential quickly became negative and gave a steady reading in an hour's time at − 0·334 v. 1 cc. of this solution was withdrawn with a clean pipette, taking precautions not to admit oxygen, and was placed in a vacuum tube (specially cleaned as described by Harrison [1927]), together with 0·1 cc. of 1/5000 solution of methylene blue. The reduction time *in vacuo* at 25° was then determined. It was found to be 50 minutes. To the solution remaining in the electrode vessel was added 0·025 cc. of copper sulphate solution, containing 1/200 mg. copper, making the final concentration of copper in solution approximately 1/4000 mg. per cc. The reduction potential became slightly more positive and in half an hour's time was steady at − 0·318 v. 1 cc. of this solution was then withdrawn, placed as before in a specially cleaned vacuum tube, with 0·1 cc. 1/5000 methylene blue solution and the reduction time was again determined. This was found to be $3\frac{1}{2}$ minutes.

Thus the trace of copper decreased the reduction time from 50 to $3\frac{1}{2}$ minutes without effecting any increase in the negative reduction potential of the cysteine solution.

This experiment has been repeated a number of times, always with a similar result.

The effect of the ferric ion is similar to that of the cupric ion, *i.e.* a slightly more positive potential is obtained at equilibrium, whilst a catalytic action is observed. The catalytic effect of iron is much less marked than that of copper, this having already been observed by Harrison [1927].

Ferrous ions have a reverse effect to ferric and cupric ions, a slightly more negative reduction potential being observed on their addition. Since the object of these experiments was to determine whether the catalytic effect of traces of metals was necessarily connected with any change in reduction potential brought about by the metals, a mixture of ferrous ions and cupric ions was added to the metal-free cysteine solution in the electrode vessel. Cupric ions were used in preference to ferric ions because of their powerful catalytic action. It was found that a mixture of ferrous and cupric ions, consisting of 1/100 mg. iron (as ferrous ammonium sulphate) and 1/150 mg. copper (as copper sulphate) effected no change, within experimental limits of error, in the reduction potential of cysteine. For example, in one experiment the initial reading (with $N/1000$ cysteine solution at 18°) was − 0·303 v. and after addition of the mixture of metal ions given above, the steady reading in an hour's time was − 0·301 v. As before, when the steady potentials were obtained, 1 cc. of the solution was withdrawn, before and after the addition of the metals, and the reduction times of each solution were determined. The

reduction time of 0·1 cc. 1/5000 methylene blue solution before metal addition was 22 mins. at 19° and after metal addition 3 mins. at 19°.

The reduction experiments were performed at the same time, the first solution being stored in pure nitrogen until the second solution was withdrawn.

In another experiment the initial steady potential at 18° of $N/1000$ cysteine solution previous to the addition of a mixture of ferrous and cupric ions was − 0·294 v. and, after addition of the metal mixture, the steady potential was also − 0·294 v. (The addition to this solution of a further quantity of cysteine increased the negative reduction potential by an amount equal to that required by the equation obtained by Dixon and Quastel [1923], connecting the potential with the concentration of cysteine.)

The experiments we have described indicate that traces of metals, which bring about a marked catalytic effect with cysteine, do not bring about the increase in negative reduction potential which is demanded on Dixon's hypothesis.

Summary.

Metal-free cysteine gives at a gold electrode a high negative reduction potential, which is not increased by the addition of traces of catalysts such as ferric or cupric ions. This is contrary to the result which would be expected on Dixon's hypothesis of the mechanism by which the reduction potential of cysteine is secured at the electrode.

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LXXXIV. SOME PROPERTIES OF THE DEHYDROGENATING ENZYMES OF BACTERIA.

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Introduction.

It has been shown on the activation theory put forward by one of us [Quastel, 1926] that not only are many of the phenomena of biological oxidations and reductions brought into line with one another and made interpretable on a common basis, but that the relative effects of the introduction of various groups into a particular substrate on the hydrogen-donating or -accepting power of the latter can be qualitatively estimated. For instance it can be shown that the introduction of a CH_3 group into glycollic acid should produce a substance (lactic acid) which would be a more powerful hydrogen donator than glycollic acid, whilst the introduction of a COOH group should produce a substance (hydroxymalonic acid) which would have feebler hydrogen-donating powers than glycollic acid towards the same hydrogen acceptor (methylene blue) and in the presence of the same activating system. Again the relative effects of the introduction of CH_3 or OH or $CH_2.COOH$ into succinic acid and other substances can be qualitatively estimated, and the predicted results are found to be in agreement with the experimental.

In this examination, however, of the properties of substances on the activation theory it has been tacitly assumed that all the substances in question are accessible to or adsorbed by the active centres or the enzymes, and the facts that results predicted on the activation theory are borne out by experiment, and that no instance has yet been found which is contrary to the expected result, are in favour of the truth of this assumption. Yet it may be maintained that this agreement between predicted and experimental result is entirely fortuitous. If it can be shown by experiment, however, that the substrates whose properties we are investigating (whether they be activated or not) are actually adsorbed by certain active centres, this will provide strong support of the active centre hypothesis and will render much more remote the possibility that the agreement between the experimental results and those predicted on the theory of activation is simply a coincidence.

The aim of the investigation to be described in this paper has been to ascertain

(1) whether the assumption is correct that a number of substrates, for which results were predicted on the activation theory and were confirmed by experiment, are actually adsorbed at active centres (whether they are activated or not);

(2) whether any particular type of compound is adsorbed at one centre rather than at another.

We should expect *a priori* that a centre possessing certain groups would adsorb a particular type of compound and that another centre with different groups would adsorb a different type of compound, *i.e.* the active centres would evince a *specificity of adsorption*. But of the total number of molecules capable of being adsorbed at a particular centre we expect only a few to be activated. The number of these would depend upon the strength and nature of the polarising field and the structure of the substrate molecules. Thus there are two factors which limit the specificity of action of a centre (or enzyme), (*a*) the specificity of adsorption at the centre, (*b*) the structure of the adsorbed molecules in relation to the nature of the activating field at the centre.

Experimental procedure.

It has been shown [Quastel and Wooldridge, 1927, 1] that the effect of exposing *B. coli* to toluene is to bring about a considerable number of inactivations. The enzymes for lactic, succinic and formic acids are, however, left intact. Hence toluene-treated *B. coli* forms suitable material for determining the action on the lactic, succinic and formic acid enzymes of a number of substances, inert in presence of the treated organism but possibly active with the normal organism. The experimental procedure is to determine the time of reduction of a quantity of methylene blue by, say, lactic acid in presence of the treated organism and to compare this with the time of reduction obtained with a mixture of the lactic acid and the substance under investigation, *e.g.* oxalic acid, under exactly similar conditions. From the two times, the percentage retardation of the velocity of reduction of methylene blue by lactic acid is easily determined. The following is an example of the procedure in one experiment. In a vacuum tube (Thunberg) were placed 2 cc. phosphate buffer solution p_H 7·4, 1 cc. 1/5000 methylene blue solution, 1 cc. $M/50$ lactic acid (neutralised) solution, 2 cc. water and 1 cc. of a suspension of the toluene-treated organism. The tube was then evacuated and placed in the water-bath at 45° and the reduction time to a standard colour corresponding to 95 % reduction of the dye was determined. The reduction time was 10 minutes. In another tube was placed the same mixture of phosphate buffer solution, methylene blue, lactic acid and toluene-treated organism. But instead of water there were added 2 cc. $M/4$ oxalic acid (neutralised). The reduction time to the standard colour was determined as before. The time was 180 minutes. The percentage retardation by this con-

centration of oxalic acid ($M/14$) of the velocity of reduction by lactic acid ($M/350$) was $\frac{180-10}{180}.100 = 95\ \%$ (approx.).

In all cases substances under investigation were neutralised (if necessary) with sodium hydroxide to p_H 7·4, and solutions in the vacuum tubes were always buffered at this p_H. Reductions were always carried out at 45°.

The toluene-treated organism was prepared by shaking a suspension of freshly grown *B. coli* with toluene. Previous to treatment with toluene the freshly grown organism was washed three times with physiological saline solution, aerated for one hour, nitrogen was passed through for another hour to help to remove oxygen, and finally the suspension of the organism in saline was evacuated in order to remove gases as much as possible from the suspension. The latter precaution is necessary as it is not easy to remove the last traces of oxygen from the bacterial emulsion when it is mixed with various solutions in the vacuum tubes.

After the suspension of the organism was shaken with toluene it was allowed to stand, still in contact with toluene, at room temperature for 1 hour. The bacterial emulsion was then pipetted clear from the layer of toluene, and the emulsion was used in this state.

Reduction times were taken to a standard 95 % reduction of the methylene blue in order to eliminate the departure from the linearity of reduction velocity during the last 5 % reduction.

The experimental error in obtaining the time of reduction is about 1 to 2 minutes. Hence where retardations in the time of reduction are of this order the effects are not taken as real. Thus if there be a retardation of a normal reduction time of 20 to 22 minutes, by admixture of a substance, the percentage retardation, which is 9 %, is not regarded by us as real. It is necessary, therefore, to adjust the amount of organism so that the normal reduction time (*i.e.* the time without admixture of extraneous substances) is of the order of 15 to 25 minutes, so that the experimental error of 2 minutes gives a relatively small apparent percentage retardation (up to 14 %). Retardations greater than 20 % are real; the longer the time of retarded reduction the greater is the accuracy of observation.

Results with lactic acid (toluene-treated organism).

The percentage retardations of the velocity of reduction of methylene blue due to lactic acid by various substances are given in Table I. The following substances, among those investigated, are strongly adsorbed: glycollic acid, oxalic acid, glyoxylic acid, hydroxymalonic acid, glyceric acid, α-hydroxybutyric acid, mandelic acid, pyruvic acid, mesotartaric acid, *dl*-tartaric acid, and presumably lactic acid.

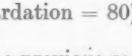
The structure common to these substances, and presumably that necessary for adsorption at the lactic acid enzyme is —CO—COH* or —CHOH—COH* where H* is mobile, *i.e.* the substance is acidic.

Table I.

Percentage retardations by various substances of the velocity of reduction of methylene blue by lactic or succinic acids (neutralised) in the presence of toluene-treated *B. coli* or an extract obtained from autolysed *B. coli*. All substances used were first adjusted to p_H 7·4 and the experiments were made *in vacuo* at 45° as described for Table II. The "toluene-treated *B. coli*" and "extract" were prepared as described in the text.

	Toluene-treated *B. coli*			Extract	
Added substance	Concentration of added substances	% retardation on succinic enzyme *M*/140 succinic acid	% retardation on lactic enzyme *M*/350 lactic acid	Concentration of added substances	% retardation on lactic enzyme *M*/70 lactic acid
α-Hydroxybutyric acid	*M*/70	—	>30	*M*/70	>30
Hydroxy*iso*butyric acid	*M*/14	0	0	—	—
Glyceric acid	*M*/28	0	>80	*M*/14	>80
Pyruvic acid	*M*/14	10	90	*M*/28	68
Alanine	*M*/14	0	25	*M*/14	21
Acetic acid	*M*/14	0	0	*M*/14	0
Glycollic acid	*M*/14	0	>80	*M*/28	>95
Glycine	*M*/14	0	25	*M*/14	21
Glycol	*M*/7	0	0	*M*/7	0
Oxalic acid	*M*/28	14	>95	*M*/28	>95
Glyoxylic acid	*M*/14	0	>82	*M*/28	>95
Malonic acid	*M*/14	>95	15	*M*/28	0
Hydroxymalonic acid	*M*/14	0	90	*M*/28	>95
Glycerol	*M*/7	0	10	*M*/28	18
Phenylacetic acid	*M*/14	77	55	*M*/7	40
Mandelic acid	*M*/14	52	76	*M*/7	>95
Phenylpropionic acid	*M*/35	>95	61	—	—
Tricarballylic acid	*M*/10·5	52	0	*M*/28	18
Citric acid	*M*/10·5	30	0	*M*/28	25
Pyrotartaric acid	*M*/7	90	50	—	—
Tartaric acid	*M*/14	10	47	*M*/14	20
Mesotartaric acid	*M*/14	50	90	*M*/28	>95
Parabanic acid	*M*/140	42	80	*M*/112	>95
Hydantoin	*M*/56	0	0	*M*/56	0
Succinic acid	—	—	—	*M*/14	0
Glutaric acid	*M*/14	43	25	—	—
Succinimide	*M*/10	0	0	—	—
Glutamic acid	*M*/14	5	27	*M*/28	0
Cyanoacetic acid	*M*/7	15	0	—	—
Thiodiglycollic acid	*M*/14	0	0	—	—
Laevulinic acid	*M*/14	0	0	—	—
Glucose	*M*/14	0	0	*M*/14	0
Mannitol	*M*/14	0	0	—	—
Formic acid	—	—	—	*M*/28	0
Fumaric acid	—	—	—	*M*/14	0
Aspartic acid	—	—	—	*M*/28	25
Malic acid	—	—	—	*M*/28	31

Acetic acid, malonic acid, glycol, glycerol, glycine, succinic acid, formic acid, hydroxy*iso*butyric acid, citric acid, etc. are either not adsorbed or only very feebly so, illustrating the specificity of adsorption for a particular *type* of structure by the lactic acid enzyme. It was interesting to determine the effect of substitution of the two OH groups of oxalic acid.

Parabanic acid was found to be powerfully adsorbed (% retardation = 80), but hydantoin  , as we would anticipate from the previous results, was not adsorbed under our experimental conditions.

The affinity of oxalic acid for the lactic acid enzyme (which does not activate oxalic acid) is very striking. A concentration of $M/7000$ will bring about a 50 % retardation of the velocity of reduction of methylene blue due to $M/140$ lactic acid.

Pyruvic acid is strongly adsorbed. Its retardation of the normal velocity of reduction is not due to its acting as a hydrogen acceptor (*i.e.* to its oxidising leucomethylene blue), for it does not appreciably retard the velocity of reduction due to succinic acid. Were it a hydrogen acceptor in presence of the toluene-treated organism, like fumaric acid, it would retard reductions both by lactic and succinic acids.

Since fumaric, malic, and aspartic acids act as hydrogen acceptors in presence of the organism they cannot be used with this organism to determine whether their adsorption occurs at the lactic acid enzyme. From other evidence we have found that neither succinic nor fumaric acid is adsorbed by this enzyme, that malic acid is adsorbed, but only to a slight extent and not nearly so powerfully as glycollic acid.

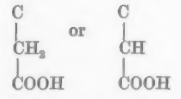
Mesotartaric and *dl*-tartaric acids are interesting in demonstrating the effect of a change in internal structure on adsorption. Mesotartaric acid is much more powerfully adsorbed than tartaric acid.

Results with succinic acid (toluene-treated organism).

The results are given in Table I. The following substances, among those investigated, are strongly adsorbed: succinic acid, pyrotartaric acid, tricarballylic acid, phenylpropionic acid, malonic acid, glutaric acid, mesotartaric acid.

The substances adsorbed by the lactic acid enzyme (with the exception of mesotartaric acid), acetic acid, glycine, glycerol, glycol or the sugars are either not adsorbed by the succinic acid enzyme or only feebly so. Phenylacetic and phenylpropionic acids are more adsorbed at the succinic acid enzyme than at the lactic acid enzyme; but parabanic acid, which has a definite effect at the succinic acid enzyme is less powerful here than with the lactic acid enzyme. The same applies to mesotartaric acid.

The structure necessary for adsorption at the succinic acid enzyme seems to be



associated possibly with another carboxyl group. Malonic acid has a very powerful affinity for the succinic acid enzyme (though it is neither a hydrogen acceptor nor donator), its action being similar to that of oxalic acid for the lactic acid enzyme. The phenyl group may also play a part in facilitating adsorption at the succinic acid enzyme. It is interesting that mandelic and hydroxymalonic acids should offer such clear contrasts to phenylacetic acid

and malonic acid respectively, in their relative effects on the succinic acid and lactic acid enzymes.

The effects of malonic acid and substituted malonic acids.

Table II shows reduction times due to lactic, succinic and formic acids in presence of toluene-treated organism and the effects on these times of the admixture, with the normal substrate, of either malonic acid, hydroxymalonic acid, or ethylmalonic acid. Results with oxalic acid are also inserted for comparison. It will be seen how specifically effective malonic acid is on the succinic acid enzyme, and hydroxymalonic and oxalic acids are on the lactic acid enzyme. The formic acid enzyme is not affected. Ethylmalonic acid has little or no effect on the three enzymes.

Table II.

Effects of the addition of certain substances upon the times of reduction of methylene blue by succinic, lactic and formic acids in the presence of toluene-treated *B. coli*. Each vacuum tube contained 2 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue solution, 1 cc. toluene-treated *B. coli*, 1 cc. of the donator at the head of each vertical column, 1 cc. of the substances, in a concentration of $M/2$, given horizontally and 1 cc. water. All the acids were brought to p_H 7·4 with sodium hydroxide. The reductions were carried out *in vacuo* at 45°. ∞ indicates that reduction was not complete in 3 hours.

Added substance, $M/14$	$t_{succinate}$, $M/140$	$t_{lactate}$, $M/350$	$t_{formate}$, $M/700$
NaCl	23′	10·7′	12′
Malonic acid	∞	14·5′	11·7′
Hydroxymalonic acid	21·3′	∞	10′
Ethylmalonic acid	20·5′	18′	10·5′
Oxalic acid	32·5′	∞	11·3′

Activations by toluene-treated B. coli.

Among the substances adsorbed by the lactic acid enzyme the following are activated as hydrogen donators by the normal organism: tartaric, glycollic, mandelic, hydroxymalonic, glyceric, α-hydroxybutyric and lactic acids. After treatment of *B. coli* with toluene (see Table III for results) the following only are activated: lactic, α-hydroxybutyric and glyceric acids. The activations of lactic and α-hydroxybutyric acids are either not at all or only relatively slightly affected by the toluene treatment whilst that for glyceric acid ($M/28$) seems to be decidedly affected. Glyceric acid is interesting in that its activation does not appear to be inhibited to the same extent by the presence of oxalic acid ($M/14$) as are those of α-hydroxybutyric and lactic acids. It is possible that the explanation will be found in a consideration of the relative adsorption coefficients of these substances at the lactic acid enzyme. As we have shown in earlier papers toluene effects the elimination of a number of dehydrogenating enzymes, including those for the sugars and glutamic acid.

The lower fatty acids.

In earlier papers we considered that the activation of acetic acid ran parallel with that for formic acid. This appears to be an error. Normal *B. coli* activates formic, acetic and propionic acids [Quastel and Whetham, 1925] but after its treatment with toluene the activations of acetic and pro-

pionic acids no longer occur although that for formic acid is left intact. The fact that we obtained positive results for acetic acid with toluene-treated organism we suspect to be due to our sample of acetic acid (*Kahlbaum 100 %*) probably containing formic acid to the extent of 1 in 5000 (which would be sufficient to account for the result) rather than to the fact that we are now working with a different strain of organism. Our present sample of acetic acid effects no reduction of methylene blue in presence of the toluene-treated organism although it will do so in presence of the normal organism[1] (Table III). Acetic acid will not retard the time of reduction due to formic acid on admixture with the latter. As stated earlier the formic acid enzyme seems to be independent of the substances we have yet investigated.

Table III.

Effects of toluene upon the activation of certain donators by *B. coli*. The toluene-treated *B. coli* was prepared from the normal *B. coli*. Conditions as in Table II.

Substance	Reduction times with normal *B. coli*	Reduction times with toluene-treated *B. coli*
None (control)	100′	∞
Succinic acid, *M*/140	14′	12′
Lactic acid, *M*/350	7′	7′
α-Hydroxybutyric acid, *M*/35	6·5′	9·7′
Glyceric acid, *M*/14	15·5′	71′
Glycollic acid, *M*/14	24′	∞
Mandelic acid, *M*/14	73′	∞
Hydroxymalonic acid, *M*/7	40′	∞
Tartaric acid, *M*/7	21′	∞
Glutamic acid, *M*/14	17′	∞
Acetic acid, *M*/7	24′	∞
Glucose, *M*/140	7′	∞

The action of malonic acid and oxalic acid on the activation of succinic acid and lactic acid respectively (toluene-treated B. coli).

Figs. 1 and 2 show quantitatively the effect of malonic acid at various concentrations on the reduction of methylene blue in presence of a constant quantity (*M*/140) of succinic acid, and of succinic acid at various concentrations in presence of a constant quantity of malonic acid.

Figs. 3 and 4 are similar for lactic and oxalic acids. The succinic and lactic acids are at such concentrations (in Figs. 1 and 3) that increase of these does not appreciably increase the velocity of reduction.

The curves show (*a*) that the effects of malonic and oxalic acids are reversible; (*b*) that malonic and oxalic acids do not inhibit at rates directly proportional to their concentrations but at rates which we would anticipate if they were adsorbed at the enzymes. Sufficient quantitative work has not yet been done to be certain of the exact form of the curves. They appear to follow approximately the logarithmic function found by Quastel and Whetham [1925] to hold for the relationship between velocity of reduction and concentration of substrate.

[1] To perceive the effect of acetic acid it is necessary to use relatively large quantities of the *freshly grown* organism.

Results with normal B. coli.

Owing to the activity of many substances which are rendered inert by treating *B. coli* with toluene, results obtained by using mixtures of substrates with the normal organism are not so clearly defined as those with the treated organism. It has been possible to show, however [Quastel and Wooldridge, 1927, 2], that the substrates do not all act independently of one another—as they should if entirely distinct enzymes (one for each substrate) were

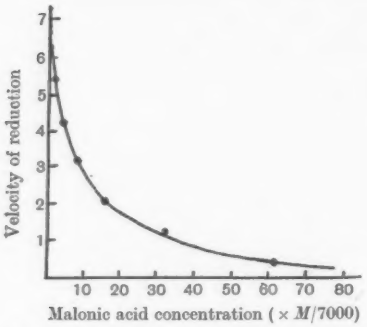


Fig. 1. The effect of variation in malonic acid concentration on the velocity of reduction due to a constant quantity (M/140) of succinic acid.

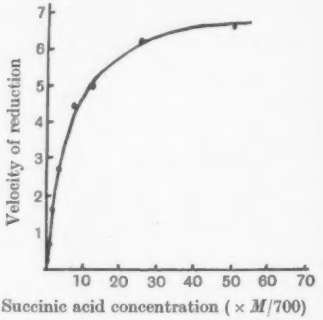


Fig. 2. The effect of variation in succinic acid concentration on the velocity of reduction in presence of a constant quantity (M/700) of malonic acid.

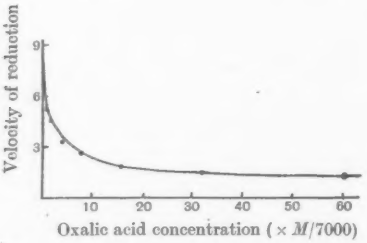


Fig. 3. The effect of variation in oxalic acid concentration on the velocity of reduction due to a constant quantity (M/140) of lactic acid.

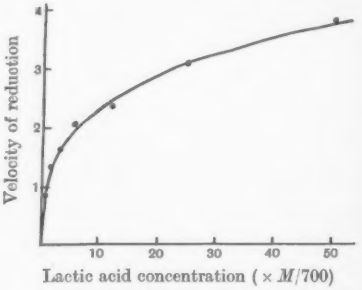


Fig. 4. The effect of variation in lactic acid concentration on the velocity of reduction in presence of a constant quantity (M/700) of oxalic acid.

involved. This was clearly shown in the case of the sugars, where a mixture of two different sugars at their saturation concentrations reduced at a rate either intermediate between the rates of the sugars taken separately or at a rate equal to that of one of them. The enzymes for glucose and for formic acid were clearly independent of one another, but in most other instances

"overlapping" occurred which could only be interpreted by assuming that adsorptions of substrates occurred at enzymes not concerned with their activations. The effects of treating the organism in various ways were examined and it was found that a treated organism did not necessarily have the same power of adsorbing a substrate at an enzyme as it had previous to treatment—a result which we would expect on the view of active centres but which is difficult to reconcile with the view that enzymic activity is due to the effects of specifically active molecules adsorbed at a surface. Results with the normal organism (Table IV) show that the effect of oxalic acid on the lactic

Table IV.

Rates of reduction due to donators A, and mixtures of the donators with other substances B. t_A, t_B and t_{A+B} = times of reduction in mins. respectively due to A, B or $A+B$ in the presence of "resting" *B. coli*. Conditions as in Table II.

A	B (M/14)	t_A	t_B	t_{A+B}
Lactic acid, M/350	Malonic acid	12·3	∞	16
"	Hydroxymalonic acid	"	53	18
"	Ethylmalonic acid	"	∞	13·7
"	Oxalic acid	"	"	25
"	Mandelic acid	10	"	18
Succinic acid, M/140	Malonic acid	21	"	58·3
"	Hydroxymalonic acid	"	53	15
"	Ethylmalonic acid	"	∞	22
"	Oxalic acid	"	"	25
Glucose, M/140	Malonic acid	7·7	"	9
"	Hydroxymalonic acid	"	53	9·3
"	Oxalic acid	"	∞	8·5
"	Mannitol	"	10	8
Glutamic acid, M/14	Malonic acid	18·3	∞	23·3
"	Hydroxymalonic acid	"	53	17
"	Oxalic acid	"	∞	17·3
Formic acid, M/700	Malonic acid	7	"	7
"	Hydroxymalonic acid	"	53	7
"	Oxalic acid	"	∞	6·3
α-Hydroxybutyric acid, M/35	Malonic acid	7·7	"	7
"	Hydroxymalonic acid	"	"	10·5
"	Oxalic acid	"	"	12
"	Mandelic acid	"	"	12
Glyceric acid, M/14	Malonic acid	15·5	"	17
"	Hydroxymalonic acid	"	53	13·7
"	Oxalic acid	"	∞	14·3
"	Mandelic acid	"	"	18·3

acid enzyme is less for equivalent concentrations than with the toluene-treated organism. How general this phenomenon is and what the correct interpretation of the action of toluene is, have yet to be determined. But it would appear that toluene can not only eliminate the activations of a number of substrates which are adsorbed at a particular enzyme or centre (and which are still adsorbed after the eliminations have been effected) but can also change the affinity or adsorbing power of the enzyme for substrates.

Considering further the results with the normal organism it is interesting (Table IV) that neither oxalic acid nor hydroxymalonic acid affects perceptibly the activation of glucose. This shows quite conclusively that the reduction of methylene blue by glucose in presence of *B. coli* does not occur through the

intermediate formation of lactic acid. This acid is undoubtedly formed from glucose in the presence of *B. coli* but the reducing power of glucose on methylene blue is independent of the production of lactic acid.

Malonic acid has apparently an inhibitive effect on the activation of glutamic acid.

The effects of toluene.

The fact that not only toluene but many other capillary-active substances (*e.g.* benzene, ether, acetone, chloroform, ethyl and propyl alcohols) bring about similar inactivations leads us to consider the possibility that the inactivations are due to the association of these substances with certain groups, probably non-polar groups, in the active centres. We have been able to show that at the lactic acid enzyme a number of substrates, *e.g.* glycollic, mandelic, tartaric acids, which are hydrogen donators with normal *B. coli* but are inactive after treatment of the organism with toluene, are adsorbed, whether the organism has been treated with toluene (Table I) or not (Table IV). We have also been able to show that the adsorption of a substrate, *e.g.* oxalic acid, at the lactic acid enzyme differs quantitatively in the case of toluene-treated *B. coli* and in that of the normal organism (see Table V). This is explicable on the active centre hypothesis, for the association of certain groups of the lactic acid centre with toluene will not only result in a change of the adsorption coefficients of the centre for the various substrates capable of being adsorbed at it, but also alter the nature and strength of the polarising field, so that substrates previously activated after being adsorbed may no longer be activated although they are still adsorbed.

Table V.

Reduction times of lactic acid with various concentrations of oxalic acid both in the presence of normal "resting" *B. coli* and with toluene-treated *B. coli*. Conditions as in Table II.

Mixture	Normal *B. coli*	Toluene-treated *B. coli*
Lactic acid, *M*/140 (alone)	10·5′	8·5′
,, + oxalic acid, *M*/114688	11·3′	10′
,, + ,, *M*/28672	12·5′	13·3′
,, + ,, *M*/7168	15′	23·7′
,, + ,, *M*/1792	15·5′	48′
,, + ,, *M*/448	18·5′	72′
,, + ,, *M*/112	19·3′	95′
,, + ,, *M*/28	17′	141′

A possible argument for the mode of action of toluene is that this substance not only inactivates a number of enzymes, but also renders the cell more permeable to substrates such as, for instance, oxalic acid. Thus the argument would be that the *entire* lactic acid enzyme is inside the cell which is normally only slightly permeable to oxalic acid and that the effect of toluene is to enter the cell, inactivate a large number of enzymes and render the cell more permeable to oxalic acid, which now, because of its greater concentration within the cell, has a greater effect on the lactic acid activation. We have

already shown in previous publications that permeability considerations will not account for our results, and that indeed [Quastel, 1926] the main site of reduction of methylene blue is at the cell surface. But it is possible finally to dismiss the above argument by the following experiment. Small concentrations of oxalic acid are nearly as effective quantitatively as large concentrations on the rate of reduction of methylene blue by lactic acid with the normal organism (Table VI). Now we would expect, since the velocity of diffusion of a substance into the cell is dependent on its concentration outside, that large quantities of oxalic acid would be much more effective than small concentrations in bringing about a given retardation of the velocity of reduction due to lactic acid. Hence since this is not the case we must conclude that the velocity of diffusion of oxalic acid into the normal cell is comparatively quick and the lactic acid enzyme soon becomes "saturated" with regard to oxalic acid whether the concentration of the latter outside the cell be large or small. On leaving the normal bacteria in contact with oxalic acid (neutralised) at various concentrations for 2 hours at room temperature in the absence of lactic acid and methylene blue, we expect that, at the end of this time, the concentration of oxalic acid inside the cell will have increased, and hence that if the lactic acid enzyme was not quickly "saturated" such saturation, or at any rate a much greater adsorption of oxalic acid at the enzyme, will have been achieved during the 2 hours' interval. Experiment (Table VI) shows that at the end of the 2 hours the various concentrations of oxalic acid show no greater retardations of the velocity of reduction due to lactic acid than in the former case where there had been no special interval allowed for adsorption to occur. Our only conclusion must be that the lactic acid enzyme is quickly fully "saturated" with regard to oxalic acid. The retardation of velocity of reduction effected is of the order of 70 %. Yet when the organism is treated with toluene the retardation effected, under precisely similar conditions, is well over 90 %. Thus permeability considerations do not explain the phenomena and the only satisfactory explanation seems to us at present to be that based on active centres.

Table VI.

Reduction times of lactic acid with various concentrations of oxalic acid in the presence of "resting" *B. coli*. In column *A* the times given were obtained as described in Table II. Column *B* gives results obtained after first allowing the organism to stand in the presence of the neutralised oxalic acid and buffer for 2 hours at room temperature before adding the lactic acid and methylene blue.

	A	*B*
Lactic acid, *M*/350 (alone)	18·5′	15′
„ + oxalic acid, *M*/840	49·6′	42′
„ + „ *M*/420	61·7′	43′
„ + „ *M*/210	56·3′	52·5′
„ + „ *M*/105	55·5′	56·5′

A CONSEQUENCE OF THE ACTIVE CENTRE HYPOTHESIS.

We should be able to demonstrate on the active centre theory that if enzymes drawn from different sources can activate a particular substrate they should all have the power of adsorbing a particular type of compound of which the active substrate in question is an example.

Stephenson [1928] has reported that if a suspension of *B. coli* be allowed to autolyse at an alkaline reaction (in the presence of phosphates as buffer) for several days at 37°, and the suspension be then centrifuged, the clear supernatant liquid contains materials which can activate lactic acid as a hydrogen donator.

The enzymes extracted in this way can bring about only the activation of lactic and α-hydroxybutyric acids and also to a very slight extent of glyceric acid.

Table I gives the effects of admixture of various substances on the velocity of reduction due to lactic acid in presence of the extract, and it will be noted that precisely the same type of substances effects large retardations of the velocity of reduction with the extracted lactic acid enzyme as that which is effective with the toluene-treated organism. Thus in spite of the fact that there is apparently a considerable difference in physical condition of the lactic acid enzyme in the toluene-treated organism from that of the extracted "soluble" lactic acid enzyme (which can be shown, however, to be heterogeneous), the properties of the two enzymes with reference to the specific adsorption of substances of the lactic acid type are the same.

It seems reasonable to suspect that the process of extraction of the enzyme (carried out by autolysis at an alkaline reaction for several days) has effected an alteration of the lactic acid centre of the normal organism in a manner similar perhaps to that effected by toluene, and it is only this altered structure which can be dissociated from the remainder of the organism and be examined in a "soluble" (though heterogeneous) condition. Of course there may be a variety of centres to which the lactic acid type of compound is accessible and these differ in their activating powers. The structure which is extractable is the one which can only effect very few activations. Whether this view is right or not it seems to us worth pointing out that treatment of a cell may affect in some way, perhaps only a minor one, the properties of the enzymes of the cell, so that although an examination of the properties of the enzyme extracted from the cell by certain means must be invaluable in indicating its nature and structure, yet the means by which the enzyme was obtained must be considered in determining the properties of the enzyme in its initial condition in the cell.

So far as we can ascertain from our investigation there seem to exist centres which adsorb specifically substances of the succinic acid type, others which adsorb specifically those of the lactic acid type, those which adsorb specifically those of the glucose-mannitol type and those which adsorb only

formic acid. Yet with the normal or untreated organism many of the centres may contain groupings common to each other which result in "overlapping" [Quastel and Wooldridge, 1927, 1, 2]; the effect of treatment is to render the centres more distinct from each other so that less of this "overlapping" occurs and it is easier to determine what type of structure is accessible to one centre and what to another.

Just as has been shown by Coombs [1927] in the case of the xanthine oxidase, there is a considerable specificity of adsorption by the dehydrogenating enzymes of bacteria for substrates. Out of the number of compounds adsorbed specifically at each enzyme or active centre, only a number can be activated and these are just those whose relative activities can be predicted on the basis of the activation theory.

Summary.

1. The enzyme (or active centre) of *B. coli*, which activates lactic acid as a hydrogen donator has the property of specifically adsorbing compounds characterised by the possession of a particular structure which seems to be —CO—COH*— or —CHOH—COH* where H* is mobile, the compound having acidic properties. The specificity of adsorption is very marked.

2. The enzyme (or active centre) which activates succinic acid has also the property of adsorbing compounds characterised by the possession of a particular structure. This seems to be —C—CH—COOH or —C—CH_2—COOH.

3. The formic acid enzyme is independent of the lower fatty acids (other than formic acid itself) and of all the substances we have so far investigated.

4. The activity of glucose as a hydrogen donator is not perceptibly inhibited by the presence of oxalic acid, or of hydroxymalonic acid. The reduction of methylene blue by glucose in presence of bacteria is independent, therefore, of the intermediate production of lactic acid.

5. The action of toluene on *B. coli* is to bring about an elimination of the activity of the organism to many substances, which can still be shown to be adsorbed at an active centre. Toluene appears also to affect the adsorption coefficient of the active centre towards substrates. The effect of toluene is interpretable on the basis of the active centre hypothesis, but difficult to understand on other views.

6. Using a "soluble" preparation of the lactic acid enzyme, it has been shown that this preparation has precisely the same specific adsorbing power on a particular type of compound, as has the lactic acid enzyme of *B. coli*. The activations of the "soluble" preparation are the same as those of the lactic acid enzyme of *B. coli* after treatment with toluene, and the significance of this is discussed.

These observations support the active centre hypothesis of enzyme action.

We are very grateful and much indebted to Sir F. G. Hopkins for the interest he has taken in this work and to Prof. M. A. Whiteley for supplying us with some of the substances used in this investigation.

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LXXXV. THE INHIBITORY EFFECT OF SUGARS ON HAEMOLYSIS BY SODIUM TAUROCHOLATE.

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The effect of certain sugars on saponin haemolysis has already been studied by quantitative methods [Ponder and Kennedy, 1926]. This study has shown that glucose, mannose, lactose, sucrose, trehalose, maltose, raffinose, arabinose, and rhamnose are inhibitory in different degrees when human red cells are used in the haemolytic systems, and that the action of these various sugars on the human red cell presents quantitative differences from the action on the cells of the sheep. In no case is the inhibition great, the value of R rarely exceeding 1·5. It has been further shown [Ponder, 1926] that the greater part of the action of the inhibitory sugars is upon the cells themselves rather than upon the saponin contained in the haemolytic system, for 90 % of the inhibitory effect remains after the cells have been treated with the sugar and afterwards subjected to washing. There is also some evidence that the sugars have an effect on the lysin in these systems, although this effect is very small compared with that on the cells themselves. We have no information, however, as to whether this latter inhibition is attributable to the presence of the sugar or to the fact that the addition of sugar to the system involves the removal of NaCl from it.

This investigation is concerned with the much more difficult problem of the inhibitory action of the sugars in systems containing sodium taurocholate as the lysin.

Materials used.

The sugars whose action we have investigated (glucose, sucrose, and lactose) were chemically pure specimens, and were dissolved in water to make $M/3{\cdot}6$ solutions, such solutions being isotonic with the cells employed. It is desirable that these shall be freshly prepared.

The red cells used were those of man. A suspension was prepared by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of 0·85 % NaCl, the blood being first received into citrate. Such a suspension is identical with that used in our previous investigations.

The sodium taurocholate used in these experiments was a highly purified and carefully dried sample. The use of this salt as a haemolytic agent neces-

sitates the greatest care in the preparation of the series of dilutions. The taurocholate is first weighed out in a dry weighing bottle, and dissolved in sufficient 0·85 % NaCl to make a 2·5 % solution; this is done about half an hour before the experiments are to be commenced. Immediately before a dilution of the substance is required for producing lysis, 1 cc. of this 2·5 % solution is diluted as required, the dilution being carried out in such a way that the final concentration of lysin in the completed system (0·8 cc. of lysin, 0·8 cc. of saline or sugar solution, and 0·4 cc. of suspension) is 1 in 1000, 1 in 2000, etc. In this way the dilute solutions of the lysin are prepared immediately before use, this method of preparation taking advantage of the fact that the salt is stable in concentrated solutions, but reducing to a minimum the effects of the instability which is met with in dilute solutions. If the dilute solutions are prepared and allowed to stand, it will be found impossible to obtain consistent readings.

When it is necessary, as it is in the experiments to be described below, to plot several time-dilution curves for several haemolytic systems which are afterwards to be compared, the plotting of these curves must be proceeded with in a special manner. A dilution of lysin, say 1 in 1000, is prepared, and immediately after the preparation the haemolytic activity of this dilution in each one of the haemolytic systems is ascertained. In this way several points are obtained, each corresponding to a dilution of 1 in 1000, but each lying on a different curve. The next dilution, say 1 in 2000, is then prepared in a similar manner from the 2·5 % stock solution of taurocholate, and its activity measured in each one of the systems; this results in a second series of points, each belonging to a different curve, being obtained: and so on until the curves are completed. This procedure differs from that usually employed when saponin is used as a lysin, for in the latter case each time-dilution curve may be plotted separately, and completed before any points on the time-dilution curves for the related systems are obtained. The special procedure to be used in conjunction with sodium taurocholate is necessary in order to obviate errors arising from instability of the haemolysin. A similar procedure, it may be mentioned, should be used when sodium oleate is employed as a lysin.

The 0·85 % NaCl used in the experiments should be freshly prepared with conductivity water which has not been exposed to the air for any considerable period. If the saline becomes acid through the taking up of CO_2 the acidity alters the activity of the sodium taurocholate. For many purposes it is desirable to use a buffered saline, but it must be remembered that the introduction of the buffers complicates the haemolytic system and may alter some of the properties of the lysins.

Results.

The results of this investigation will be best expressed by commencing with the analysis of two typical experiments.

Exp. 1. Time-dilution curves were plotted for two haemolytic systems at 25°, the first system (Curve A) containing 0·8 cc. of taurocholate (various dilutions), 0·8 cc. of saline, and 0·4 cc. of suspension, and the second system (Curve B) containing 0·8 cc. of taurocholate (various dilutions), 0·8 cc. of $M/3{\cdot}6$ sucrose, and 0·4 cc. of suspension. The time-dilution curves are shown in Fig. 1, and below are figures for the usual analysis:

c_1	c_2	R
4·0	2·66	1·50
2·0	0·60	3·33
1·0	0·53	1·88
0·66	0·48	1·39
0·50	0·40	1·25
0·40	0·32	1·25

It will be observed, from inspection of the plotted curves, that this result is very different from that obtained when saponin is used as a lysin in conjunction with sucrose as an inhibitor, for the time-dilution curve for the taurocholate-sucrose-cell system shows a bulge in the region of 1 in 1000 to

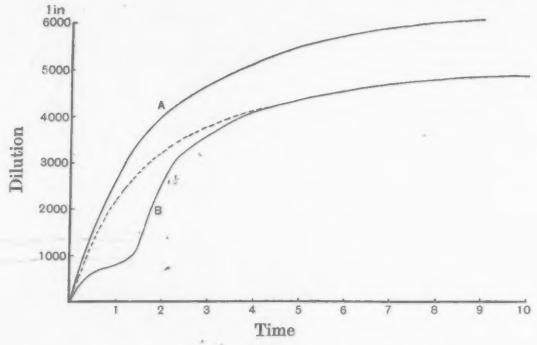

Fig. 1.

1 in 2000 dilutions of the lysin. This peculiarity is reflected in the variable value of R (equal to c_1/c_2), and it will be recalled that in saponin-sucrose-cell systems the value of R is constant. Were R constant in the case of the system under consideration, the curve for the taurocholate-sucrose-cell system would be that indicated by the dotted line in Fig. 1.

To explain this anomalous result, let us assume that the sucrose has a double action on the system, affecting the cells so as to make them more resistant to the lysin, and at the same time depressing the activity of the lysin itself. Indications of this double action have already been described in the case of saponin-sucrose-cell systems [Ponder, 1926], and the remarkable depression of the activity of the lysin which occurs when either saponin or sodium taurocholate acts in NaCl-free systems is an additional fact in favour of its occurrence. Let us further assume that the activity of the lysin is depressed to a negligible extent in systems in which it is present in asymptotic

concentration. Then the inhibitory effect of the sucrose on the cells of the system will be

$$\frac{c_{1\infty}}{c_{2\infty}} = R_\infty$$

which is equal to 1·25 in this particular experiment; *i.e.* the sucrose so affects the cells that it makes them 1·25 times as resistant to the lysin as normally. If we further call the activity of the lysin I, the activity in the absence of any depression due to the sugar being unity, we have

$$\frac{R_\infty}{R} = I,$$

where the values of R are those given in the table above, and are determined from the ratio c_1/c_2. We may now tabulate the values of I, the activity of the lysin, for various concentrations:

c_1	I
4·0	0·83
2·0	0·38
1·0	0·66
0·66	0·90
0·50	1·00
0·40	1·00

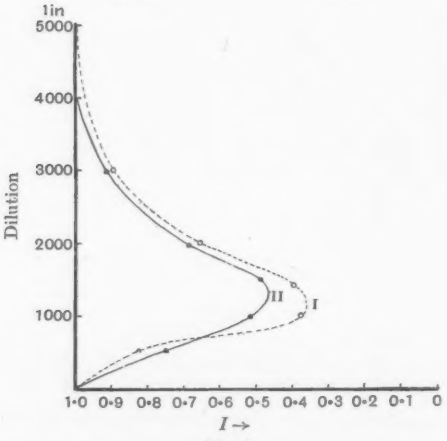

Fig. 2.

Plotting these values, we obtain Curve I in Fig. 2. The activity of the lysin is depressed to the greatest extent when the lysin concentration is about 1 in 1000; when the lysin is either very dilute or very concentrated, the activity is scarcely depressed at all.

Exp. 2. In making the above analysis, we have assumed the existence of a double action on the part of the sucrose, the cells being rendered more resistant by exposure to it, and the activity of the lysin being at the same time depressed. There fortunately exists a method by which we can demonstrate these two effects separately [Ponder, 1926], and this experiment is concerned with its application.

Three preparations are made from the standard suspension of cells. Suspension 1 is obtained by diluting 5 cc. of this standard suspension with 10 cc. of saline. Suspension 2 is prepared by adding 10 cc. of $M/3{\cdot}6$ sucrose to 5 cc. of the suspension, and allowing the mixture to stand about half an hour before use. Suspension 3 is made by adding to 5 cc. of the standard suspension 10 cc. of $M/3{\cdot}6$ sucrose, and allowing the mixture to stand at 25° for 30 minutes. 10 cc. of the mixture are then transferred to a small volumetric flask; the contents of this are then distributed into two centrifuge tubes, each of 10 cc. capacity, and 5 cc. of saline added to each. The contents of the tubes are centrifuged, the supernatant fluid is pipetted off, and the cells, together with the small amount of fluid which remains, are replaced in the volumetric flask. The cells are washed out with several small additions of saline, and the washings added to the flask. The 10 cc. volume is then made up with saline, the cells thoroughly distributed throughout the fluid, and the suspension which results used in the experiments. It consists of cells which have been treated with sucrose, but at the same time these cells are suspended in saline. It follows from the method of preparation that suspensions 1, 2 and 3 are of the same cell concentration. Further, if we prepare a system containing 0·8 cc. of lysin and 1·2 cc. of either suspension 1, 2 or 3, we have the reacting components in the same concentration as in the system 0·8 cc. of lysin, 0·4 cc. of standard suspension, and 0·8 cc. of saline or sucrose as the case may be.

Three time-dilution curves are now plotted. Curve 1, Fig. 3, is that for taurocholate acting on the untreated cell suspension (suspension 1). Curve 2, Fig. 3, is for taurocholate acting on cells which have been treated with sucrose and then washed, and Curve 3, Fig. 3, is for a taurocholate-sucrose-system (suspension 3), as in the first experiment of this paper. The figures of the analysis are given below:

	Curve 2		Curve 3		
c_1	c_2	R	c_2	R	I
4·00	2·87	1·39	2·50	1·60	0·75
2·00	1·66	1·20	0·87	2·29	0·52
1·33	1·11	1·20	0·64	2·46	0·49
1·00	0·83	1·20	0·57	1·75	0·69
0·66	0·55	1·21	0·51	1·30	0·92
0·50	0·425	1·17	0·42	1·19	1·00

The result of this experiment, in which the action of the sucrose on the cells (Curve 2) is investigated separately from the action on the lysin itself (obtained from the analysis of Curve 3 in terms of Curves 1 and 2), justifies the assumption made in Exp. 1 that the sucrose has a double action, increasing the resistance of the cells on the one hand and depressing the activity of the lysin on the other. In this particular experiment, the resistance of the cells is increased 1·2 times, while the extent to which the activity of the lysin is depressed in various concentrations may be seen from an inspection of the values of I, plotted in Curve II of Fig. 2. It will be observed that the two curves showing the relation of I to the concentration of lysin, and derived

from the two different experiments, are very similar in general appearance; the value of I is at a minimum, and the depression of the activity of the lysin at a maximum, in the neighbourhood of the concentrations 1 in 1000 and 1 in 2000. When the lysin is either very concentrated or very dilute, the activity is little altered by the presence of the sucrose.

The fact that the depression of activity of the lysin is at a maximum in concentrations of 1 in 1000 to 1 in 2000 is a suggestive one, for it is well known that it is in these concentrations that sodium taurocholate is the least stable. This instability in these concentrations is frequently very obvious when time-dilution curves are plotted for the lysin acting on washed red cells, for correct time readings are much more difficult to obtain for the concentrations between 1 in 1000 and 1 in 2000 than for other concentrations. Indeed, if a large number of time readings are made for these and intermediate concentrations, it will be found that the points do not lie on the curve as they

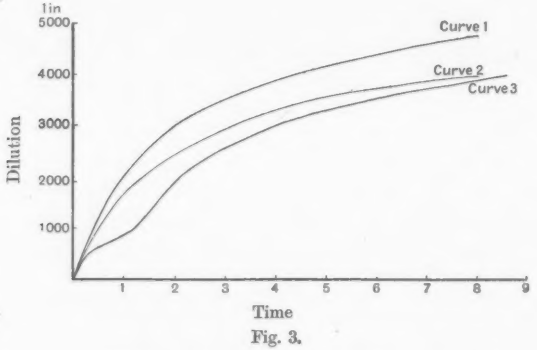

Fig. 3.

do for other concentrations, but that the curve in this region passes through a cluster of points. Some of the points in the cluster lie on the curve, and these are presumably the correct ones, the others being evidence of the instability of the lysin in this region, and of the great difficulty which is experienced in obtaining consistent readings. This same difficulty has been noted by Shattuck [1928], working with sodium taurocholate and chicken cells. When the activity of taurocholate is depressed by sucrose and other sugars, the greatest depression apparently takes place in this very region, *i.e.* in the region where the lysin is most unstable.

This effect is not produced by sucrose alone, but also by glucose and lactose, and probably by all inhibitory sugars. The three sugars investigated are remarkably alike in their action both on the cells of the haemolytic systems and on the haemolysin, and the way in which I varies with the concentration is much the same in the three cases. The maximum depression always occurs in the concentrations from 1 in 1000 to 1 in 2000, and the way in which I varies with the concentration of lysin is very much the same for

each of the three sugars. In the following table are given the results of three experiments which illustrate the similarity. The figures in the columns are values of I:

c	Sucrose $R=1{\cdot}25$	Glucose $R=1{\cdot}24$	Lactose $R=1{\cdot}23$
4·00	—	0·83	0·62
2·00	0·40	0·52	0·62
1·00	0·62	0·55	0·51
0·66	0·77	0·75	0·85
0·50	0·91	0·90	0·95
0·40	0·94	1·00	1·00
0·33	1·00	—	1·00

As a result of these experiments, we are entitled to conclude that the sugars have a double inhibitory action in taurocholate-sugar-cell systems, one part of the action being on the cells, and the other part on the lysin itself. It will, of course, suggest itself that an increase in the resistance of the cells to the lysin, and a diminution of the activity of the lysin itself, are two terms which, regarded from the point of view of the final effect, have the same meaning; the justification for separating the two effects of the inhibitor is, however, that the effects can be separated experimentally in the manner above described. In the case of saponin, with which we have already dealt, the evidence for the double action of the inhibitory sugars is very much less convincing, although it nevertheless exists, for the diminution in the activity of the lysin is one which is both small in degree and independent of the lysin concentration. There seems, however, to be justification for concluding (i) that the inhibitory action of the sugars is a double one both in the case of saponin-sugar-cell systems and taurocholate-sugar-cell systems, (ii) that in the case of the former system the principal effect is on the cells themselves rather than on the activity of the lysin, while in the latter systems the effect on the lysin may be very much greater than that on the cells themselves, and (iii) that the greatest depression of the activity of the lysin in taurocholate-sugar-cell systems occurs when the lysin is in those concentrations in which it is most unstable.

We do not feel justified in offering any suggestions as to the mechanism of the inhibitory effects. As in the case of saponin, we are confronted with the possibility that the inhibition arises not only from the presence of sucrose or other sugars in the system, but also from the fact that the introduction of these substances leaves the system poor in NaCl. It is certainly the case that when NaCl is removed from the systems altogether, as when the cells and lysin are prepared in isotonic sucrose, the haemolytic activity of saponin and of sodium taurocholate is very much reduced. It is probable that this great reduction in the haemolytic activity of the lysins under these circumstances is to be explained as the result of a direct action of the sugar on the lysin itself, as well as upon the cells which the system contains.

SUMMARY.

1. The inhibitory action of sucrose, glucose, and lactose on sodium taurocholate haemolysis is studied quantitatively, and the inhibition shown to be due to a double effect of the added sugar, one effect being to increase the resistance of the cells, and the other effect being to depress the activity of the lysin. These two effects are distinguished because it is possible to distinguish them experimentally.

2. The activity of the lysin is most depressed in those concentrations in which it is commonly found to be most unstable.

This investigation is a continuation of one carried out under a grant from the Moray Fund of the University of Edinburgh.

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LXXXVI. A MICRO-METHOD FOR THE COLORIMETRIC DETERMINATION OF UREA IN BLOOD.

By FLORENCE BEATTIE.

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(*Received March 22nd, 1928.*)

THE method is based on the gravimetric method of Fosse, Robyn and François [1914] in which urea is precipitated as dixanthylurea from deproteinised blood-serum by the addition of an alcoholic solution of xanthhydrol. Fosse's method has been carried out on a micro-scale by Nicloux and Welter [1921], and was found to give reliable results for quantities of urea as small as 0·05 mg.

In the following method the dixanthylurea precipitate was prepared according to Nicloux and Welter, but, instead of being weighed, it was dissolved in sulphuric acid (50 % by volume) in which xanthhydrol and its compounds dissolve, producing an intense yellow colour. This colour reaction is of an intensity which makes it possible to detect differences of 0·14 mg. of dixanthylurea, or in terms of urea, 0·02 mg. per 100 cc. 50 % sulphuric acid.

METHOD.

0·5–1·0 cc. of blood is deproteinised by the method of Folin-Wu.

To 1 vol. of blood add 7 vols. of distilled water, 1 vol. of 10 % sodium tungstate and 1 vol. of 2/3 *N* sulphuric acid. Shake and filter.

Place 1 cc. of the filtrate (= 0·1 cc. blood) in a centrifuge tube.

Add 1 cc. of glacial acetic acid, and 0·2 cc. of a solution of 5 % xanthhydrol in methyl alcohol.

Allow this to stand for 5 minutes. Then stir it with a glass rod, and allow it to settle again for half to one hour, or centrifuge for 20–30 minutes.

(When the quantity of urea is small, 20 mg. per 100 cc. of blood or less, the longer time is necessary to ensure complete precipitation.)

Filter off the precipitate by suction through a small Gooch crucible packed with asbestos.

Wash alternately three times with 2 cc. of methyl alcohol and distilled water, preferably saturated with dixanthylurea.

During the washing with methyl alcohol detach the pump until the methyl alcohol has almost drained through, to allow of complete solution of the excess xanthhydrol.

Test the third methyl alcohol washing with 50 % sulphuric acid. If no yellow colour appears, the washing is complete.

Wash out the flask and place a small receiving tube inside so as to catch the succeeding filtrate. Pour about 5 cc. of 50 % sulphuric acid into the crucible.

When the precipitate is dissolved, attach the pump, and draw the yellow

solution into the receiving tube. Wash with a further 4 cc. of sulphuric acid. Remove the tube and make up to 10 cc. in a graduated test-tube. Compare in a colorimeter with a standard solution.

The precipitate can be filtered, washed, and dissolved in sulphuric acid in about 10 minutes.

Preparation of standard solution. Dilute 0·4 cc. of 0·01 % urea solution to 1 cc. with distilled water. The same procedure is then adopted as in the case of blood-filtrate, *i.e.* 1 cc. urea solution, 1 cc. glacial acetic acid, 0·2 cc. 5 % methyl alcohol solution of xanthhydrol. The precipitate is centrifuged, filtered, washed and dissolved in 10 cc. of 50 % sulphuric acid.

This standard corresponds to 0·04 mg. urea in the 10 cc. of acid used. The standard retains its colour indefinitely.

In cases in which the blood-urea is markedly increased, the solution may be diluted with 50 % sulphuric acid until the colour is near that of the standard.

Calculation. $\frac{10}{x} \times 40 =$ mg. urea per 100 cc. blood; where standard is set at 10 mm. and $x =$ length of column of solution of unknown concentration.

When the unknown solution has been diluted, $\frac{y}{x} \times 40 =$ mg. urea per 100 cc. blood; where $y =$ no. of cc. to which unknown solution has been diluted.

Some results obtained by this method are given below:

(*a*) for solutions of known urea concentration;

(*b*) blood-urea values obtained by this method, compared with results obtained by the urease-aeration method of Marshall [1913].

(*a*) *Urea in mg. per* 100 *cc. solution.*

Theoretical	Xanthhydrol colorimetric method
45	46
55	54
25	25
30	30
35	33
80	81
100	99

(*b*) *Urea in mg. per* 100 *cc. blood.*

	Urease method	Xanthhydrol colorimetric method
Rabbit	40	39·6
Sheep	44·8	45
Human blood, from cases of renal inefficiency	46	46
	70	69
	71	71
	52	50·6
	58	59
Human blood (normal)	36	35
	30	29·3
	29·5	30
	28	29

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LXXXVII. VITAMIN D IN ADULTS.

ITS EFFECT ON THE CALCIUM AND INORGANIC PHOSPHATE OF THE BLOOD.

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(*Received March 29th, 1928.*)

Hess and Lundagen [1922] showed that in summer there is a seasonal increase of the inorganic phosphate in the blood of infants. The same phenomenon was observed in adults by Havard and Reay [1925] who found a change from 2·9 mg. per 100 cc. in winter to 4·0 mg. per 100 cc. in summer. This has been confirmed recently by Pucher [1927]. It has been suggested that this increase is due to the greater incidence of ultra-violet light during the summer months. A similar seasonal variation has been noted to occur in the serum calcium and inorganic phosphate in rabbits [Grant and Gates, 1925]. In human beings however no seasonal change in the serum calcium has been observed, and from the narrow limits within which the normal values lie it is unlikely that this should occur.

Recent work by many authors, notably Rosenheim and Webster [1927] has made it very probable that the healing of rickets by ultra-violet light, which is accompanied by a rise in the inorganic phosphate of the blood and sometimes of the calcium of the serum, is due to the formation of vitamin D from the sterols of the epidermis. This suggested that the relatively low level of the inorganic phosphate of the blood during the winter is due to a slight deficiency of vitamin D, while during the summer the level is raised owing to increased formation of vitamin D by the greater incidence of ultra-violet light. But it is difficult to accept the suggestion that vitamin D, which is concerned primarily with bone formation, should affect the calcium phosphate metabolism of adults, in whom presumably the composition of the bones is constant.

Hess and Sherman [1927] found no rise in the serum calcium after feeding irradiated cholesterol to normal adult dogs. Kultjugin [1927] irradiated rabbits with ultra-violet light and found a rise in the blood inorganic phosphate and a decrease in serum calcium. Kroetz [1927], administering irradiated ergosterol in amounts above the therapeutic dose, in human adults obtained a decrease in blood inorganic phosphate and a slight fall or no change in the serum calcium. With irradiation he found a rise in phosphate and the K/Ca ratio. The position then is clearly unsatisfactory.

Experimental.

(A). *Irradiated ergosterol feeding.*

Irradiated ergosterol was obtained in the form of "Radiostol" pellets from the British Drug Houses. Dr Underhill, of this firm, reported from feeding experiments that 1 mg. of the irradiated ergosterol from which our batch had been prepared was equivalent to more than 50 cc. of cod-liver oil.

Two experimental subjects were given 4 mg. daily, by mouth, for 11 and 13 days respectively, and a third was given 8 mg. daily for 21 days. These doses are comparable to those found by György [1927], Prinke [1927], Beumer and Falkenheim [1927] and others to be adequate in rickets. A fourth subject was kept throughout as a control. The serum calcium (Clark and Collip's modification [1925] of the Kramer-Tisdall [1921] method) and inorganic phosphate of the blood (Briggs's [1922] method) were determined daily for a control period of a week before the feeding began and daily estimations were continued during the course of the experiment. All blood samples were taken in the mornings and after not less than one hour's rest. The values varied considerably from day to day, especially in the case of the inorganic phosphate, but no significant changes in any one direction were found during the experimental period. This is shown in Table I.

Table I.

Inorganic phosphate of whole blood, and serum calcium, before and during (A) administration of irradiated ergosterol, (B) carbon-arc irradiation. (Note that the differences between the mean values for control and experimental periods are of the same order as the "probable error of the mean value" and negligible compared with the seasonal change.)

		Inorganic phosphate of whole blood					Serum calcium				
Subject	Experimental conditions	No. of determinations	Mean values mg. P per 100 cc.	Difference in mean values	Probable error of mean*	Maximum percentage range	No. of determinations	Mean values mg. Ca per 100 cc.	Difference in mean values	Probable error of mean*	Maximum percentage range
(A). *Administration of irradiated ergosterol:*											
R. E. Havard (M.)	Control period	9	2·98	0·13	0·05	23	4	9·58	0·01	0·04	2·4
	4 mg. *per diem* irradiated ergosterol	11	3·11		0·02	14	10	9·59		0·05	7·0
E. Howland (F.)	Control period	8	3·76	0·01	0·03	10	3	9·93	0·05	0·05	2·5
	4 mg. *per diem* irradiated ergosterol	12	3·77		0·03	13	11	9·88		0·05	7·7
J. O. Girsavicius (M.)	Control period	10	3·13	0·02	0·05	26	7	10·30	0·27	0·10†	9·7
	8 mg. *per diem* irradiated ergosterol	17	3·11		0·03	20	16	10·03		0·05	11·0
L. H. Stickland (M.)	Control A	9	3·16	0·04	0·05	18	4	10·06	0·40	0·21	6·6
	Control B	10	3·20		0·03	14	10	9·66		0·14	5·4
(B). *Carbon-arc irradiation:*											
R. E. Havard (M.)	Control period‡	20	3·05	0·11	0·02	23	14	9·59	Nil	0·04	7·0
	Irradiation period (16 days)	15	2·94		0·06	40	12	9·59		0·04	6·7
L. H. Stickland (M.)	Control period	19	3·18	0·09	0·02	19	14	9·81	0·13	0·05	9·8
	Irradiation period (16 days)	15	3·09		0·03	15	13	9·94		0·06	9·8

* For formula see Hill, Long and Lupton [1924].
† Several of these readings were done on 1 cc. serum with a resulting experimental error of about 3 %.
‡ This includes the period of irradiated ergosterol feeding.

(B). *Carbon-arc irradiation.*

Two subjects were irradiated daily, except on Saturdays and Sundays, for a total period of 16 days[1]. Under these conditions, this would have been sufficient to give considerable improvement in cases of clinical rickets [Hess and Unger, 1922]. General irradiation was used from three carbon-arc "white flame" lamps each about 4 ft. distant. For rickets, under these conditions, 20 minutes' exposure twice a week would have been given. In these experiments we began with 5 minutes' exposure and increased this by 5 minutes daily to a maximum of 30 minutes. Desquamation and pigmentation were produced by the end of the experiments. Daily phosphate and calcium estimations were made. No significant changes were found (see Table I).

Conclusions.

These experiments failed to demonstrate any change in the winter levels of the blood inorganic phosphate and serum calcium. It seems unlikely from these negative results that the winter level of the blood inorganic phosphate is associated with any relative lack of vitamin D in our experimental subjects. Further they cast doubt, at any rate in the case of adults, upon the accuracy of the conception that the higher value of the blood inorganic phosphate during summer is due to the increased incidence of ultra-violet light.

Summary.

1. Addition of 8 mg. per day of irradiated ergosterol to the diet of a healthy adult for 21 days during the winter caused no significant change in the blood inorganic phosphate or serum calcium.

2. Irradiation by carbon-arc lamps for a period of 16 days during winter caused no change in the blood inorganic phosphate or serum calcium.

3. Doubt is cast on the conception that the summer rise in the level of the inorganic phosphate of the blood in adults is due to increased incidence of ultra-violet light.

Our sincerest thanks are due to Mr J. B. S. Haldane, who suggested this work to us, and to Sir F. G. Hopkins and Prof. W. E. Dixon for much helpful criticism and advice. We wish to thank also our experimental subjects for their self-sacrificing co-operation.

[1] We are indebted to Addenbrooke's Hospital, Cambridge, for the use of lamps.

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LXXXVIII. NOTE ON THE CALIBRATION OF BARCROFT MANOMETERS[1].

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(*Received April 3rd, 1928.*)

The extensive use of manometric methods in studying processes involving gas exchanges, carried out largely with the type of manometer employed by Warburg, makes a simple method of calibrating the apparatus desirable. The method proposed by Warburg [1923] is accurate only to a few per cent. Recourse may be had to the accurate but tedious method of determining capacity by weighing the vessels (bound to the manometer) filled with mercury, several weighings and some dismantling of the apparatus being necessary.

The method proposed by Warburg can however be rendered more accurate by a more exact consideration of the conditions. The discussion by Rona and Nicolai [1926] is incomplete.

Details of the apparatus are given by Warburg [1923, 1926]. It is sufficient here to emphasise that the manometer in question is not a differential manometer, but a constant volume manometer, the level of the manometric liquid being adjustable by means of a reservoir.

It is desired to determine the capacity V of the vessel bound to the manometer, including that of the capillary down to a level M on the manometer, it being intended always to keep the level at M when the manometer is in use. The vessel, containing a cc. of water, is bound to the manometer, and is then allowed to reach equilibrium in a thermostat at $\theta°$ K. (say, 37·5° C.) by allowing the manometer to swing to and fro for 15–20 minutes.

The level of the manometric liquid (Brodie's fluid, of which 10,000 mm. equal one atmosphere) is allowed to become steady at a suitable mark below M (on that arm of the U-tube attached to the vessel). The barometric pressure $p_{\text{bar.}}$ and the room temperature T (° K.) are observed. The difference h_1 between the pressures inside and outside the vessel is measured (in mm.). As slight changes in temperature and barometric pressure take place during the measurements, these are allowed for by having in the thermostat a similar vessel bound to its manometer, in which no changes of volume are made, and subtracting from h_1 and subsequent readings the changes in manometric readings shown by this "control" vessel. We understand values of h_1 (h_1 being a mean of several readings) and subsequent readings to have been corrected in this way.

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell, of the University of Liverpool).

When h_1 has been obtained, the manometric liquid is forced up to the level M, and after 10–15 minutes' shaking the difference between pressures inside and outside is again measured (h_2), several readings being made.

The same operations are repeated after the volume of water in the vessel has been increased to a'. (The volumes a and a' should be chosen so that $V - a'$ is about one-half of $V - a$.) Measurements of h_1' and h_2' are made, with the same levels on the arm bound to the manometer as before. The room temperature T' is measured. (Any change in barometric pressure is taken account of by the change in the reading of the "control" vessel, which is left in the thermostat.)

We can now calculate the value of V.

Let the initial volume be $V - a + x$, the air pressure in the vessel being p_1. x is a quantity representing the air contained in the manometer capillary between the level M and the initial level.

Then
$$p_1 = p_{\text{bar.}} + h_1 - p_{H_2O},$$
where p_{H_2O} is the vapour pressure of water at the temperature $\theta°$ K. The volume of the air in the vessel is now decreased to $(V - a)$. Then p_1 increases to p_2, and
$$p_2 - p_1 = h_2 - h_1 = h.$$
From gas theory,
$$h = p_1 \frac{x}{V - a}.$$
Allowance must however be made for the influence of the change in pressure on the solubility of air in the water contained in the vessel.

Let α be the Bunsen coefficient for air. (At 37·5° C. α for nitrogen is 0·0135 and for oxygen 0·0242, hence for air α is 0·0156.)

It can be shown that the corrected formula is
$$h = \frac{p_1 x}{V - a\left(1 - \alpha \frac{\theta}{273}\right)}.$$
Similarly
$$h' = \frac{p_1' x'}{V - a'\left(1 - \alpha \frac{\theta}{273}\right)}.$$
Now x and x' represent the same volume and therefore when allowance is made for any change in room temperature:
$$x/x' = T'/T.$$
Combining the above three equations, we find:
$$V = \frac{\left(1 - \alpha \frac{\theta}{273}\right)\left(\frac{Tha}{p_1} - \frac{T'h'a'}{p_1'}\right)}{\frac{Th}{p_1} - \frac{T'h'}{p_1'}}.$$
A further correction should be made for the expansion of water (a and a' terms) from room temperature to $\theta°$ K. Assuming room temperature to be 15° C.

for this purpose, the expansion to 37·5° C. is from 1 to 1·0060, and when the thermostat temperature is 37·5° C., we have

$$V = \frac{0{\cdot}988\left(\frac{Tha}{p_1} - \frac{T'h'a'}{p_1'}\right)}{\frac{Th}{p_1} - \frac{T'h'}{p_1'}}.$$

An illustrative set of values are given below:

a, a'.	2·50, 8·01.
h_1, h_1'.	+ 5, − 9.
h, h'.	71·9, 139·7.
T, T'.	289·8, 290·2.
$p_{\text{bar.}}$	755/0·076.
p_{H_2O}.	48·4/0·076.
p_1, p_1'.	707·0/0·076, 705·9/0·076.

Hence it was found that $V = 13{\cdot}58$ cc.

A comparison was made of the values obtained by this method and by the method of weighing. The results on four different manometers and vessels, given below, show that the manometric method is accurate to about 1 %.

V (manometrically)	V (weighing)	Difference %
13·67	13·54	+1·0
13·66	13·68	−0·1
13·10	13·21	−0·9
13·74	13·91	−1·2

It should be noted that for accurate results h' should be as large as possible.

Summary.

A revised and simple method, accurate to about 1 %, of calibrating the Barcroft constant-volume manometer is described.

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LXXXIX. THE EFFECT OF METALLIC SALTS ON THE GLYCOLYSIS AND RESPIRATION OF TISSUES[1].

By MAURICE JOWETT and JOHN BROOKS.

From the Muspratt Laboratory of Physical and Electro-Chemistry, University of Liverpool.

(*Received April 3rd, 1928.*)

The importance of glycolysis in the metabolism of malignant tissues has been established by the work of Warburg and his collaborators [1924–1927]. Warburg has at the same time developed satisfactory methods of measuring *in vitro* the respiration and glycolysis occurring in tissues. By glycolysis is meant the splitting of glucose to lactic acid.

Warburg's findings may be summarised in the statements: (*a*) glycolysis under *anaerobic* conditions takes place to any considerable extent only in growing tissues, and (*b*) glycolysis under *aerobic* conditions takes place to any considerable extent only in malignant tissues. Glycolysis under aerobic conditions is always less than under anaerobic, respiration having the power of lessening glycolysis by an amount in proportion to its own magnitude, this effect being termed the Pasteur reaction.

These *in vitro* findings have been confirmed by the work of Rona and Deutsch [1926] and of Murphy and Hawkins [1925]. Confirmatory work *in vivo* has been carried out by Tadenuma, Hotta and Homma [1924], Cori and Cori [1925], and by Warburg, Wind and Negelein [1926].

The importance of these results for the problems of malignant disease suggested that an investigation of the effects of some heavy metal salts on glycolysis and respiration in tissues would be of interest, especially in view of the use of lead compounds as therapeutic agents in cases of malignant disease by Blair Bell *et al.* [1926].

The present paper is an account of such an investigation. At least one result of importance has been obtained, namely that several metallic salts have a poisoning action on glycolysis and respiration which is progressive with time, the poisoning proceeding as a unimolecular reaction. The rate of the metabolic process thus decreases as an exponential function of the time. Such an exponential effect, so far as we are aware, has not hitherto been

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell, University of Liverpool).

described for the action of poisons on any enzyme process, whether intracellular or extracellular.

Rahn [1916], in discussing theoretically the effect of poisons on enzyme processes, suggested that poisons would have a dual action, accelerating both the rate of enzyme action and the rate of enzyme decomposition, so that an initial acceleration of the process would be followed by retardation owing to inactivation of the enzyme. It was suggested that the enzyme concentration would decrease exponentially with time, but no quantitative evidence was brought forward to support this hypothesis. It was also suggested that the accelerative phase would be greater in dilute solutions of the poison. These views form a quantitative expression of the so-called Arndt-Schulz law, to which such work as that of Neuberg and Sandberg [1920] on yeast fermentation appears to lend support. Doubt has been cast on the reality of the accelerative phase in the action of poisons on the aerobic fermentation of yeast by the work of Meier [1926], who showed that the apparent acceleration by mercuric chloride is due to the neglected effect of the poison on respiration. Schoeller and Gehrke [1926] have however found alkaloids to exert at low concentrations a stimulating effect on the anaerobic glycolysis of yeast. While in the present work we have not found accelerations by metallic salts in most cases, lead appears able to produce accelerations.

Other workers on the action of heavy metals on enzyme processes, whether extracellular [Ollson, 1921] or intracellular [Meier, 1926], have found the effect of metallic poisons to be progressive with time, but the effects do not appear to be an exponential function of time.

Cook [1926] studied the effect of salts of heavy metals on the respiration of *Aspergillus niger*. His results show a time-effect of poisons on the rate which follows a formula for the concentration of an intermediary product in consecutive reactions, but his theoretical considerations appear to be unsound. A latent period was observed, and sometimes an initial acceleration.

The most serious attempt to analyse the effect of heavy metals on enzyme processes appears to be that of Euler and Svanberg [1919] and Euler and Walles [1924], who found the action of heavy metal salts on saccharase, whether isolated or within the cell, to be very different from that found by us for the processes studied in this paper. An initial poisoning was observed, which disappeared with the passage of time, the enzyme being gradually regenerated.

The glycolysis and respiration of some tissues.

Although the main object of the present work has been to study the effect of metallic salts on glycolysis and respiration, some measurements of the absolute values of these metabolic rates have been made. These are summarised in Table I, as they may be of interest in connection with similar data obtained by other observers.

Table I. *Metabolism of various tissues.*

Tissue	Q_{O_2}	$Q^{O_2}_{CO_2}$	$Q^{N_2}_{CO_2}$	R.Q.	U
Rat sarcoma R 10	11	—	42	—	+20
Flexner rat carc.	6	18	28	—	+16
Carc. omentum (hum.)	—	—	13	—	—
Carc. mammae (hum.)	8	10	20	—	+ 4
Rat liver	9·7	0	1·4	0·96	−18
Rat spleen	—	—	10·0	—	—
Rat testis	—	—	9·9	—	—
Rabbit spleen	12	5	—	—	—

Q_{O_2} (Respiration) = mm.³ oxygen consumed per hr. per mg. tissue (dry weight).

$Q^{O_2}_{CO_2}$ (Aerobic glycolysis) = mm.³ carbon dioxide evolved per hr. per mg. tissue (dry weight) in presence of oxygen, beyond the evolution due to respiration.

$Q^{N_2}_{CO_2}$ (Anaerobic glycolysis) = mm.³ carbon dioxide evolved per hr. per mg. tissue (dry weight) in absence of oxygen.

The carbon dioxide evolved in glycolysis is derived from neutralisation by bicarbonate of the lactic acid formed. Warburg assumes [1927] that under limiting conditions of efficiency of the Pasteur reaction, such as may be found *in vivo*, the aerobic glycolysis will be equal to the "excess fermentation," U, which is defined by the relation

$$U = Q^{N_2}_{CO_2} - 2Q_{O_2}.$$

A large positive value of U is a characteristic of malignant tissue.

Returning now to a consideration of Table I, we should state that the values given there represent in most cases the mean values from a number of experiments. They are in the main very similar to those recorded by other workers [Cannan, 1927].

A number of experiments on the anaerobic glycolysis of rat tumours showed that the value of $Q^{N_2}_{CO_2}$ is not altogether constant[1]. Thus in a young, rapidly growing tumour (R 10) $Q^{N_2}_{CO_2}$ showed a value of 60, as against the average value of 42.

The data given for the Flexner rat carcinoma are very similar to those of other workers, those in the table being a mean from four experiments carried out in duplicate. Nine experiments on the anaerobic glycolysis of the same tumour, four or five slices being taken in each experiment, gave a mean value for $Q^{N_2}_{CO_2}$ of 32, which is practically identical with the value of other workers. Here again, however, one experiment showed a value of 53.

The human tumours reported on were diluted with non-malignant tissue.

Other workers have reported a slight aerobic glycolysis in the case of liver, but in two out of three experiments we have found none, the values of the respiratory quotient observed being 0·885, 0·955 and 1·040. The measurements refer to a one-hour period.

In the case of spleen, our results are confirmatory of those of Murphy and Hawkins [1925], who found aerobic glycolysis. This we find apparently to increase with time, which may be explained as due to a progressive damaging of the mechanism of Pasteur's reaction.

[1] For an example see Protocol 1, p. 736.

Experimental methods.

In the present work, the apparatus and methods of Warburg have been used for the measurement of respiration and glycolysis. The experimental details are fully described in Warburg's papers [1924, 1926]. The method is a manometric one[1]. A slice of the tissue under investigation, thin enough (less than 0·5 mm. in thickness) to allow adequate diffusion of the metabolites throughout, is shaken in a suitable medium, at a temperature of 37·5° and the gas exchange is observed.

Glycolysis. The medium used was either Ringer's solution or horse serum to which 0·2 % glucose had been added. The Ringer's solution was prepared by mixing isotonic solutions (*i.e.* solutions of freezing point − 0·56°) of the various salts and unless otherwise stated had the following composition:

Salt	Moles per litre
NaCl	0·139
KCl	0·0029
$CaCl_2$	0·0020
$NaHCO_3$	0·0191

The gas-mixtures used for saturating the media (and as the gas-phase in the experimental vessels) were 5 % carbon dioxide in oxygen for the measurement of aerobic glycolysis, and for anaerobic glycolysis 5 % carbon dioxide in nitrogen. The p_H of the medium is then given by the Henderson-Hasselbalch equation [Hasselbalch, 1917]. In the present case, the p_H at 37·5° was 7·35.

The quantities measured are the amounts of oxygen consumed by the tissue and the amount of carbon dioxide evolved. The latter may arise from respiration and from glycolysis (lactic acid formed liberates carbon dioxide from the bicarbonate of the medium).

The anaerobic glycolysis can be determined from an experiment with one tissue slice. For the aerobic glycolysis, it is necessary to carry out an experiment with two slices of tissue, each in its own vessel with a different volume of medium. When serum is used as medium additional experiments are required, as the formation of a given amount of lactic acid does not liberate an equivalent amount of carbon dioxide [Warburg, 1925].

Respiration. The medium used was a Locke's solution containing 0·2 % glucose, and having the following composition:

Salt	Moles per litre
NaCl	0·158
KCl	0·0033
$CaCl_2$	0·0023

The solution was saturated with oxygen, which also formed the gas-phase in the experimental vessels. The vessels have an additional compartment containing a concentrated solution of NaOH which absorbs the carbon dioxide evolved by the tissue. The quantity measured is therefore the amount of oxygen consumed by the tissue.

[1] For a method of calibration of the Barcroft manometer which was found useful, see previous paper by Jowett [1928].

In view of the sensitivity of the Pasteur reaction to experimental injury the experiments on the action of metallic salts have been confined to the effects exerted on glycolysis under anaerobic conditions, and on respiration under conditions where no glycolysis takes place (*i.e.* in Locke's solution).

Over the periods of time covered by the majority of the experiments, the tissue slices did not usually maintain a constant rate of glycolysis or respiration. A slow falling off in rate was usually observed after 30–60 minutes. For this reason the following procedure was adopted.

The rate of glycolysis or respiration of a number of slices of the tissue under investigation in different vessels was observed over a period of 30 mins. The substance to be tested was then added to several of the vessels, no addition being made to two of the vessels, which then acted as "controls." The rates were then followed for all the vessels until slices similarly treated began to show divergent behaviour or the "control" slices fell off considerably in rate.

It was assumed that the effect of the added substance on the rate of glycolysis or respiration was an effect superimposed on any spontaneous falling off in rate observed in the case of the two "control" slices, and the effect was calculated on this basis. The results were only accepted when the behaviour of duplicates was in good agreement.

The observed effects on the rate of glycolysis or respiration are considered to be accurate within 2–5 %.

The mode of addition of the substance under investigation to the vessels containing tissue slices was such that no appreciable change was brought about in the bicarbonate content or isotonicity of the medium (with the exception of the experiments where lead acetate solution was added directly to vessels containing Ringer's solution). The volume of added solution or suspension was always small, except in some experiments with calcium chloride. In those cases where a slightly soluble salt (*e.g.* $PbCO_3$) was used the solid was added directly or in the form of a suspension in the medium, while soluble salts (*e.g.*, $ZnCl_2$ or $CdCl_2$) were added in the form of a solution in the medium.

The majority of the experiments were carried out with rat tissues. Two transplantable rat tumours have been used, the Flexner-Jobling rat carcinoma and a rat sarcoma R 10. In a few cases human malignant tissues have been studied.

Experiments with mercuric halides.

Under the conditions of concentration employed in the present work, mercuric halides have proved to have the most rapid action of any of the salts studied. As experiments *in vitro* have been limited to a few hours' duration, the action of mercury salts has thus been the most amenable to quantitative investigation and analysis.

In particular, the exponential law already referred to has been most readily established for mercuric halides.

One experiment will be given in some detail to illustrate the nature of our observations in this connection. The rates of glycolysis given in Table II are stated as percentages of the initial rate (*i.e.* the rate before mercuric chloride was added to the medium)[1]. The average rate over a period is referred to the mid-point in time of the period, and the times stated are the number of minutes that have elapsed since the addition of the mercury salt. In each case a slice of rat spleen of about 5–6 mg. dry weight was shaken in about 5 cc. of Ringer's solution containing initially the stated concentration of mercuric chloride. (The concentration decreases considerably and rapidly owing to adsorption by the tissue: evidence of this will be given later.)

Table II. *Exp.* 57. *Effect of mercuric chloride on glycolysis of rat spleen.*

	M/5000 $HgCl_2$		*M*/24,000 $HgCl_2$		*M*/97,000 $HgCl_2$	
Time (mins.)	Rate observed	Rate calculated	Rate observed	Rate calculated	Rate observed	Rate calculated
25	100	84	95	93	97	97
70	53	62	78	82	93	92
115	41	45	72	73	85	88
145	37	39	67	67	86	85
208	25	24	57	56	79	79
275	21	15	49	47	79	73

The rates of glycolysis calculated and given in Table II, which are in fair agreement with the observed values, are obtained from the relation

$$V = V_0 . e^{-\alpha t},$$

where V_0 is the initial rate, V is the rate at time t (t being measured in hours), and α is a coefficient giving a measure of the effect of the poison on the rate of the process. The values of α used in the above table were 0·414, 0·166, 0·07.

In all our other experiments the effect of mercuric halides on glycolysis and respiration of tissues was found, within the experimental error, to follow this law, the values of α naturally varying from case to case. The simplest explanation of the exponential law is that the rate of action of the enzyme is unaffected by mercury except in so far as the enzyme is inactivated by the mercury, and on this assumption the inactivation is (stoicheiometrically) a unimolecular reaction.

For further analysis of the action of mercury, it must be decided which is the slower, and therefore the controlling process, (*a*) the diffusion of mercuric chloride into the tissue, or (*b*) the reaction of mercuric chloride present in the tissue with the glycolytic enzyme.

Experiments were made in which tissue-slices (of Flexner rat carcinoma) were placed for 20–30 minutes in Locke's solution at room temperature containing mercuric chloride, and, after washing, their rate of glycolysis again

[1] For fuller details see Protocol 2, p. 737.

measured in Ringer's solution containing no mercury. The rate was lower than that prior to the mercury treatment, but remained at a steady value, instead of falling off as it would have done if mercuric chloride had been present throughout.

The experiments show that the action of mercury is irreversible under our experimental conditions, but do not decide between the possibilities (*a*) and (*b*) above. A steady lower rate is consistent with either possibility.

Experiments such as the following were therefore carried out. After measuring the glycolysis of several slices of rat testis in Ringer's solution a solution of mercuric chloride in Ringer's solution was added to some of the slices (from the additional compartment with which the vessels were fitted). After 4 and 12 minutes' further shaking respectively in the thermostat, two of the slices were removed from their vessels, washed in Ringer's solution, and placed in vessels containing Ringer's solution alone, and their glycolysis was again measured. Another slice was left in contact with the Ringer's solution containing $HgCl_2$ throughout the experiment. The results obtained are given in Table III.

Table III. *Exp.* 114. *The effect of varying times of immersion in M/5000 $HgCl_2$.*

Time after addition of $HgCl_2$ (mins.)	Rates (relative to controls = 100)		
	Tissue slice in contact with $HgCl_2$ throughout	Tissue slice immersed for 4 mins.	Tissue slice immersed for 12 mins.
10–25	37	—	—
25–40	29	33	33
40–70	20	30	28
70–85	16	32	34

If a slow diffusion of mercuric chloride into the tissue were the controlling process in the inactivation, the effect of 12 minutes' immersion in $HgCl_2$ on the glycolytic rate should have been much greater than that of 4 minutes' immersion. This is evidently not the case.

The alternative view must therefore be correct, namely that diffusion of mercuric chloride into the tissue is rapid compared with the subsequent inactivation of the glycolytic enzyme.

Evidently in 4 minutes or less diffusion equilibrium is more or less set up between tissue and medium. Diffusion into thin plates has been treated by McBain [1910], and on the basis of his treatment, if we assume that mercuric chloride diffuses through tissues at the same rate as through water, it can be calculated that equilibrium will be set up in 1–2 minutes. It thus appears that mercuric chloride does diffuse through tissues about as readily as through water.

We must suppose that on removing the tissue from the mercuric chloride solution the $HgCl_2$ which is free or reversibly bound by the tissue is redistributed between the tissue-slices and the new medium, the concentration in

the tissue being very much lowered by this process, so that the glycolytic enzyme suffers little further inactivation beyond that which it has already, irreversibly, undergone.

It was mentioned earlier that a considerable proportion of the mercuric chloride added to the Ringer's solution is removed by the tissue. This is shown by such experiments as that given in Table IV, in which α, the poisoning-coefficient, is shown to be dependent on the volume of mercuric chloride solution on which the tissue-slice can draw.

Table IV. *Exp.* 123. $M/5000$ $HgCl_2$. *Glycolysis of rat spleen.*

Cc. of solution	1·9	3·8	7·6
Weight of tissue (mg.)	7·9	8·2	6·9
Cc. of solution per mg. of tissue	0·24	0·46	1·1
α	0·26	0·36	0·55

A similar experiment with rat testis showed a similar result, namely, that α increases with the volume of $M/5000$ mercuric chloride from which unit weight of tissue can take up the salt. The explanation is that after the rapid taking up of mercury the concentration in the medium is no longer $M/5000$ but lies considerably below this value, the decrease being less the larger the volume the tissue can draw from, and α being reasonably taken to depend on the *equilibrium* concentration of mercury in the medium. That is, we take it that for mercuric chloride an adsorption equilibrium is set up rapidly between tissue and medium.

The considerable effect of the amount of adsorption on α makes it necessary to be cautious in making deductions from values of α obtained for different tissues even if the same volume of $M/5000$ mercuric chloride is available per mg. of tissue, as the specific adsorption may vary from tissue to tissue. Estimates of the "sensitivity" of glycolysis in various tissues to mercury can evidently not be made in this way, except in an uncertain qualitative manner.

We do not therefore propose to report a number of experiments of this kind that we have made, but pass on to a type of experiment in which the difficulty does not arise.

In the experiments to be reported, solid mercuric iodide was added to the Ringer's solution in place of mercuric chloride, and the effect of saturated HgI_2 solution on glycolysis and respiration measured. In this way we are studying the effect of a constant concentration (or more strictly, constant thermodynamic activity) of mercuric iodide on the processes.

Mercuric iodide apparently dissolves rapidly enough to keep the media sufficiently near to saturation. This was shown in an experiment where slices of tissue placed in Ringer's solution already saturated with mercuric iodide (solid also being present), showed the same decrease in glycolysis as slices placed in Ringer's solution to which solid mercuric iodide was then added.

It should be mentioned that neither mercuric chloride nor mercuric iodide is appreciably ionised. Abegg [1903] has studied the various equilibria

concerned at 25°. On the basis of his work the figures in Table V have been calculated roughly, and they will be applicable at least in point of order of magnitude to the temperature 37·5°.

Table V. *Concentrations in moles per litre.*

	$M/5000$ $HgCl_2$ Locke	Saturated HgI_2 Locke	Saturated HgI_2 Ringer
$HgCl_2$	$0{\cdot}6 \times 10^{-4}$	$1{\cdot}2 \times 10^{-6}$	$1{\cdot}3 \times 10^{-6}$
$HgCl_4''$	$1{\cdot}4 \times 10^{-4}$	$2{\cdot}9 \times 10^{-6}$	$2{\cdot}4 \times 10^{-6}$
$HgCl'$	$1{\cdot}1 \times 10^{-10}$	$2{\cdot}0 \times 10^{-12}$	$2{\cdot}4 \times 10^{-12}$
$Hg^{\cdot\cdot}$	$2{\cdot}0 \times 10^{-17}$	$5{\cdot}0 \times 10^{-19}$	$6{\cdot}0 \times 10^{-19}$
HgI_2	—	$1{\cdot}3 \times 10^{-4}$	$1{\cdot}3 \times 10^{-4}$
HgI_4''	—	$4{\cdot}2 \times 10^{-9}$	$3{\cdot}4 \times 10^{-9}$
HgI'	—	$1{\cdot}0 \times 10^{-10}$	$1{\cdot}1 \times 10^{-10}$
I'	—	$8{\cdot}2 \times 10^{-6}$	$7{\cdot}4 \times 10^{-6}$

Evidently for the action of mercuric chloride the chemical species $HgCl_2$ and $HgCl_4''$ are important. One or both of these, perhaps rather $HgCl_2$, will be the principal form in which mercury penetrates tissues. Which form is actually responsible for the effect on the glycolytic enzyme must be left undecided, but it appears very improbable that the mercuric ion plays a part at such low concentrations.

Similarly with mercuric iodide it is the un-ionised HgI_2 which predominates in amount, and it will probably be the principal form in which mercury will penetrate tissues in our experiments. It is reasonable to suppose that adsorption equilibrium with the tissue is rapidly set up in the present case. This supposition was confirmed by an experiment of a similar nature to those given earlier for mercuric chloride. Slices of tissue were immersed for varying periods of a few minutes in a large volume of Ringer's solution saturated with HgI_2 but containing none of the solid salt. Their subsequent rate of glycolysis was fairly similar and constant when compared with that of a tissue-slice shaken all the time in Ringer's solution saturated with HgI_2.

Experiments were undertaken in which various concentrations of sodium iodide were incorporated in the Ringer's or Locke's solution (sodium iodide replacing sodium chloride). The iodide was found to have no effect on the rate of glycolysis. It has however a considerable effect on the action of mercuric iodide on glycolysis and respiration. The results are given in Table VI, in which α has the same significance as before[1].

Table VI. *Effect of mercuric iodide in presence of sodium iodide.*

		Values of α			
Exp. No.	Process	Without NaI	$M/1000$ NaI	$M/100$ NaI	$M/10$ NaI
133	Anaerobic glycolysis of rat spleen	0·8	—	1·7	—
134	Anaerobic glycolysis of rat spleen	—	1·2	1·6	2·1
137	Anaerobic glycolysis of rat testis	1·0	1·7	2·0	2·4
139	Respiration of rat testis	2·1	2·2	2·4	3·4

The effect of NaI may be explained on the basis of its effect on the concentration of HgI_4'' in the medium. The concentration of HgI_2 (calculated for

[1] See also Protocol 3 (p. 737).

25°) is throughout $1{\cdot}3 \times 10^{-4}$ molar, while as we pass from the Ringer's solution containing no added sodium iodide to that containing $M/10$ sodium iodide, the concentration of HgI_4'' increases as shown in Table VII, calculated roughly on the basis of Abegg's figures for 25°.

Table VII. *Effect of iodide ion on HgI_4''.*

Added NaI	I′	HgI_4''	$Hg^{••}$	Total concentration of Hg compounds
0	7×10^{-6}	3×10^{-9}	6×10^{-19}	$1{\cdot}3 \times 10^{-4}$
0·001	9×10^{-4}	5×10^{-5}	4×10^{-22}	$1{\cdot}8 \times 10^{-4}$
0·01	6×10^{-3}	$2{\cdot}1 \times 10^{-3}$	1×10^{-24}	$2{\cdot}2 \times 10^{-3}$
0·1	$2{\cdot}5 \times 10^{-2}$	$3{\cdot}8 \times 10^{-2}$	5×10^{-26}	$3{\cdot}8 \times 10^{-2}$

In the presence of added sodium iodide the only mercury compounds present in any appreciable amount are HgI_2 and HgI_4''. The concentration of mercuric ion decreases from a very low value to a vanishingly small one.

If we now suppose that α is a function of the total concentration of mercury compounds in the medium (HgI_2 and HgI_4'' being assumed for this purpose to be equally effective), the values of α can be approximately reproduced as a linear function of this concentration raised to the power 0·1 (the last three experiments in the table above giving values for the exponent of 0·11, 0·08 and 0·10). This exponent is a not unlikely one for the adsorption of ($HgI_2 + HgI_4''$) by tissues or by some important tissue constituent.

It is thus not improbable that α is proportional to the amount of mercury adsorbed by the tissue as a whole, by the enzyme itself, or by some structural constituent with which the enzyme is associated. Minami [1923] has made it probable, by a consideration of experiments with narcotics, that the glycolytic enzyme acts at the surface of cell-structures.

We can therefore with some justification advance a theory of the mechanism of the inactivation of the glycolytic enzyme by mercury halides. The halide is rapidly adsorbed, the adsorption with which we are concerned being either (*a*) adsorption by the enzyme itself, or (*b*) adsorption by structures lying near the enzyme. The process of inactivation then consists in either (*a*) a comparatively slow change of the mercury-enzyme adsorption compound into an inactive form of the enzyme in which the mercury is bound by an essential chemical group of the enzyme, or (*b*) a similar combination of mercury and enzyme, the mercury passing over to the enzyme from structures nearby where it is adsorbed. In either case, the rate of inactivation would be proportional to the concentration of enzyme still active, and also probably to the amount of mercury adsorbed.

This relation finds its mathematical formulation in the equation

$$-\frac{dE}{dt} = k.E.c^{1/n},$$

where E is the active mass of the enzyme at the time t, and c the concentration of mercury compounds (HgI_2 and HgI_4'') in the medium (and also, it must be assumed, in the tissue fluids), and $1/n$ is the adsorption exponent.

When integrated, this relation becomes

$$E = E_0 . e^{-kc^{1/n} t},$$

where E_0 is the active mass of enzyme when $t = 0$, *i.e.* at the time of addition of the poison. If we now take V, the rate of the process, as proportional to the active mass of the enzyme, this equation is identical with that deduced experimentally earlier in this section, provided that $\alpha = kc^{1/n}$. For ($HgI_2 + HgI_4''$) the experimental value of $1/n$ was found to be 0·1. We take it that k will be approximately constant for any one tissue.

Values of α for media saturated with mercuric iodide have been determined, and are given in Table VIII. The anaerobic glycolysis was studied in Ringer's solution, and the respiration in Locke's solution (containing no bicarbonate). That the medium has no effect on the value of α was shown by two experiments in which α for $M/5000$ mercuric chloride was measured for the respiration of rat liver both in Locke's solution and in Ringer's solution, care being taken that the weight of tissue per unit volume was kept approximately constant in each experiment. In one experiment α (Locke) was 0·45, α (Ringer) 0·44; in the other α (Locke) was 0·37, α (Ringer) 0·38. The sensitivity of the process is thus the same in the two media.

Table VIII. *Values of α for saturated HgI_2.*

Tissue	α (glycolysis)	α (respiration)
Rat sarcoma	2·8	1·3
,, testis	1·9, 1·0	2·6, 2·1
,, spleen	1·15, 0·84	1·15
,, kidney	0·9	1·1

The glycolysis of malignant tissue is evidently more sensitive to HgI_2 than that of normal tissues. Furthermore, while in the case of normal tissues respiration is a more sensitive process than glycolysis, the reverse is the case for the rat sarcoma. The experiments with mercuric chloride show the same order for the various tissues in respect of glycolysis, the tumours being the Flexner rat carcinoma and a human cancer of the breast; they also show that the respiration of testis is more sensitive than its glycolysis. In solutions of (initially) $M/100{,}000$ $HgCl_2$ slight retardation of glycolysis could be observed. With $M/1{,}000{,}000$ $HgCl_2$ no effect on the glycolysis of Flexner rat carcinoma was observed.

The effects of copper, zinc, cadmium and calcium salts on glycolysis.

The effect of several metallic salts on glycolysis has been investigated in less detail than in the case of mercury.

Copper salts. The salts used were cupric chloride and sulphate. An $M/100$ solution was diluted several times with Ringer's solution and the resulting suspension added to the experimental vessels containing tissue slices whose rate of glycolysis had previously been observed. The amounts added were such as to make the concentration of copper in the medium initially $M/5000$

if all the added precipitate had dissolved, of which we are not certain. In the experiments given in Table IX where values of α are given to two significant figures, the resulting decrease in rate certainly followed the exponential relation previously discussed. Some evidence was obtained that the values of α were not so dependent on the relative amounts of tissue and medium as in the cases where mercuric chloride was used, and hence are more comparable for different tissues.

Table IX. *Effect of M/5000 $CuCl_2$ on glycolysis.*

Tissue	α
Flexner rat carcinoma	0·3, 0·6
Carc. omentum (human)	0·58
Rat spleen	0·23, 0·22
Rat kidney	0 — 0·1

Lower concentrations of copper salts had a smaller effect on glycolysis. From these results it seems probable that copper has a greater effect on the glycolysis of malignant tissues than on that of normal tissues.

In one experiment on the respiration of rat kidney in Locke's solution α for $M/5000$ $CuCl_2$ was found to be 0·45. This suggests that, as in the case of mercury, respiration of normal tissues is more sensitive than glycolysis.

Rat testis behaves anomalously to copper salts; the exponential law is definitely not followed. After a rapid fall in the rate of glycolysis, the rate remains fairly constant, as is illustrated in Table X.

Table X. *Exp.* 136. *Effect of M/5000 $CuCl_2$ on glycolysis of rat testis.*

Time (mins.)	% rate	Time (mins.)	% rate
19	57	24	49
39	48	54	45
69	46	84	41
124	46	129	42

The effect has not been further investigated. It is the only case of those studied in which, for copper or mercury salts, the exponential law definitely does not hold. For the other tissues the more accurate the experiment appears to be, the more accurately is the law followed; in some the agreement is only moderate, but no consistent divergences have been found, such as would suggest that some other law should be preferentially adopted.

Zinc, cadmium and calcium salts. The action of the chlorides of zinc, cadmium and calcium on the anaerobic glycolysis of Flexner rat carcinoma is given in Table XI. The effects, being small, have not been studied in detail.

Table XI. *Glycolysis of Flexner rat carcinoma.*

Exp. No.	Salt	Initial concentration Moles/litre	Percentage effect on rate 0–30 mins.	30–120 mins.
39	$ZnCl_2$	0·00018	+6	−13
40	,,	,,	−1	−15
50	$CdCl_2$	0·0017	+4	−14
54	,,	0·0002	−1	− 6
42	$CaCl_2$	0·0065	−3	0
53	,,	0·0205	+2	− 2
60	,,	0·054	−1	− 4

It can be seen that zinc ion, and cadmium ion to a smaller extent, depress somewhat the rate of glycolysis. The effect becomes more considerable as time passes, and possibly follows an exponential law.

Calcium ion has no appreciable effect over a wide range of concentration (the concentration already present in the Ringer's solution is 0·0022 *M*). Our result differs from that of Waterman [1924], who found that calcium salts depressed tumour glycolysis considerably. In our experiments we maintained the bicarbonate concentration of the medium at a constant value when any appreciable volume of isotonic calcium chloride was added. Neglect of this or other measures might give false results.

The effect of lead salts on glycolysis.

The effects of lead salts on glycolysis (and respiration) have been found to be small over the short periods of time necessarily employed. For this reason it was impossible to study in any detail the change in effect with time.

A series of experiments was carried out in which solutions of lead acetate were added to the experimental vessels containing slices of tumour tissue (suspended in Ringer's solution) whose glycolysis had previously been measured. With Ringer's solution as medium the p_H and bicarbonate concentration necessarily limit the concentration of lead ion attainable in the experimental vessels to a very small value.

The amount of lead acetate added was always in excess of the solubility of lead carbonate, which was precipitated. The p_H and bicarbonate concentration of the medium were thereby decreased, which leads *per se* to a decrease in the rate of glycolysis. The effect of varying these two factors on the anaerobic glycolysis of Flexner rat carcinoma has been determined quantitatively by Warburg, Posener and Negelein [1924]. Corrections calculated from their data were applied to the observed rates for (*a*) lowering of p_H and [$NaHCO_3$] due to addition of lead acetate; (*b*) lowering of p_H and [$NaHCO_3$] due to the production of lactic acid by glycolysis (a small correction).

Table XII. *Exp.* 18. *Flexner rat carcinoma.*

Volume of medium 5·0 cc. To each vessel was added 0·55 cc. of the following solutions after 30 mins.

	Lead acetate			
	M/50	*M*/500	*M*/2000	Water
Observed $Q_{CO_2}^{N_2}$ before addition	23·0	22·8	30·8	23·7
Observed $Q_{CO_2}^{N_2}$ after addition	17·7	19·2	27·0	20·0
Corrected $Q_{CO_2}^{N_2}$ before addition	23·2	23·0	31·1	23·8
Corrected $Q_{CO_2}^{N_2}$ after addition	22·0	20·1	27·9	20·4
Percentage change in $Q_{CO_2}^{N_2}$ (calculated from corrected values)	− 5	−12·5	−10	−14
Percentage change in $Q_{CO_2}^{N_2}$ relative to "control"	+11	+ 2	+ 5	—

The corrected rates of glycolysis refer therefore to the initial conditions of p_H and bicarbonate concentration, in these cases 7·23 and 0·0143 *M* respectively, and any alteration observed can be attributed to the specific effect of lead. The results of a typical experiment are given in Table XII.

The effect of lead is taken to be given by the percentage change in the corrected rates relative to the control. It will be seen that the rate of glycolysis is slightly accelerated by the addition of lead acetate. This result has been confirmed by other experiments which are summarised in Table XIII. The amounts of lead acetate added were similar to those given in the experiment quoted above. The experiments in the table are arranged in order of the p_H and calculated lead ion concentration[1] obtained after addition of lead acetate. The change in rate refers to the total change observed over the period investigated after the addition, which in no case exceeded 90 minutes.

Table XIII.

Exp. No.	p_H	Concentration of lead ion Moles/litre × 10^7	Tumour	Percentage effect on the rate of anaerobic glycolysis
18	7·20	1·0	F.R.C.	+ 5
18	7·19	1·0	F.R.C.	+ 2
23	7·18	1·1	R 10	+24
21	7·14	1·3	R 10	− 5
21	7·13	1·4	R 10	+ 9
18	7·06	1·9	F.R.C.	+11
23	7·01	2·4	R 10	+15
24	7·00	2·5	R 10	+27
16	6·96	3·1	F.R.C.	+33
21	6·93	3·6	R 10	+16

It will be seen that, although the observed accelerations are somewhat irregular, they are roughly proportional to the lead ion concentration.

These results are open to objection on the grounds that the corrections applied were often large and may have been somewhat in error. Experiments were therefore undertaken which should be free from this objection, the lead being added in the form of a suspension of lead carbonate in Ringer's solution.

The $PbCO_3$ suspension was prepared by adding 1 cc. *M*/100 lead acetate to 4 cc. Ringer's solution and a sufficient volume of this suspension was added to the experimental vessel to make the total amount of lead present equal to that in a *M*/5000 solution. In these experiments a Ringer's solution with a bicarbonate concentration of 0·0102 *M* was used, of which the p_H was 7·08. The resulting lead ion concentration in the experimental vessels would therefore be approximately $1{\cdot}7 \times 10^{-7}$ *M*.

In three cases horse serum was used as medium, and to the experimental vessels was added a mixture prepared by adding 1 cc. *M*/100 lead acetate to 4 cc. serum. The lead ion concentration would be rather less than the value in Ringer's solution, corresponding to the smaller solubility of lead phosphate

[1] These values are calculated on the basis of the data on the solubility of lead carbonate of Pleissner [1907].

in serum. The total amount of lead present was equivalent to $M/5000$ in each case in the experimental vessels.

The results of these experiments are summarised in Table XIV. The percentage change observed in the rate of glycolysis is given for two consecutive periods after the addition of lead carbonate (with the exception of Exp. 31).

Table XIV. *Effect of lead carbonate on anaerobic glycolysis.*

For Ringer's solution $[NaHCO_3]=0{\cdot}0102\ M.$, $p_H=7{\cdot}08$, $[Pb^{\cdot\cdot}]=ca.\ 1{\cdot}7\times10^{-7}\ M.$

Exp. No.	Tissue	Medium	% change in rate after addition of $PbCO_3$ 0–30 mins.	% change in rate after addition of $PbCO_3$ 30–120 mins.
31	F.R.C.	Ringer	—	+28 (0–120 mins.)
37	,,	,,	+ 8	0
38	,,	,,	+13	—
65	,,	,,	+ 4	+2
34	,,	Serum	+26	—
43	,,	,,	+35	+7
47	,,	,,	+ 7	−8
58	Rat spleen	Ringer	− 2	−7
66	,,	,,	+ 8	−4

The results are in agreement with those previously obtained. Lead causes an acceleration of the glycolysis of Flexner rat carcinoma in both Ringer's solution and serum. This acceleration becomes smaller as time passes and in one case (Exp. 47) changes to a retardation. The experiments with spleen show smaller effects of lead but the general behaviour is similar, the acceleration passing over more quickly to a retardation.

These results are of interest in connection with the view previously referred to that an enzyme poison at low concentrations acting over a short period of time may produce acceleration of the process, the effect becoming a retardation as time or concentration (or both) are increased.

In attempting to obtain further information on this point the impossibility was encountered of raising the lead ion concentration in the Ringer's solution to a much higher value and still maintaining conditions (of p_H and $[NaHCO_3]$) under which glycolysis could take place. The following procedure was therefore adopted. The anaerobic glycolysis of a number of tissue slices was measured in the usual way over a 30-minute period. Some of these slices were removed from the experimental vessels and, after washing in Locke's solution, immersed for a given period in 10 cc. of Locke's solution (at room temperature) containing various concentrations of lead acetate. After washing in Locke's solution and Ringer's solution their glycolysis was again measured. At least two control slices were treated in a similar way, the Locke's solution in which they were immersed containing no lead acetate. In the experiments given in Table XV the time of immersion was 30 minutes in each case. The times subsequent to immersion over which the glycolysis was measured varied from 45 to 75 minutes.

Table XV. *Effect of immersion in lead acetate (in Locke's solution) on subsequent anaerobic glycolysis.*

Exp. No.	Tissue	Concentration of lead acetate	Percentage effect on rate
87	F.R.C.	*M*/1000	−25
96	,,	*M*/2000	−14
83	,,	*M*/5000	−13
87	Rat spleen	*M*/1000	+ 5
86	,,	*M*/2000	− 5
85	,,	*M*/2000	+ 6
103	Rat testis	*M*/1000, *M*/3000, *M*/10,000	− 2, +1, −1
118	,,	*M*/1000, *M*/3000, *M*/10,000	− 4, −8, 0

It will be seen that relatively large concentrations of lead ion bring about an appreciable retardation in the subsequent rate of glycolysis of tumour tissue. Rat spleen and rat testis are evidently less sensitive to the action of lead ion, the effects lying within the experimental error.

These results are consistent with the view discussed in the introduction, that an enzyme poison which in small concentrations causes an acceleration of the process may, when its concentration is greatly increased, give rise to a retardation.

The effect of lead salts on respiration.

The effect of lead acetate on the respiration of various tissues has been studied. With Locke's solution as medium, it was possible to obtain relatively high concentrations of lead ion. The p_H of the Locke's solution used (*ca.* 7·0) was slightly decreased by the addition of the more concentrated solutions of lead acetate.

The results obtained are summarised in Table XVI. The percentage change in rate refers to the total change observed over the period investigated after the addition of lead acetate (40 to 100 mins.).

Table XVI. *Effect of lead acetate on the respiration of various tissues in Locke's solution.*

Exp. No.	Tissue	Initial concentration of lead acetate in Locke's solution	Percentage change in rate
93	Carc. mammae (human)	*M*/5000	+10
97	Sarc. shoulder (human)	*M*/5000	0
98	Sarc. groin (human)	*M*/5000	+ 4
94	Rat spleen	*M*/2500, *M*/5000	− 6, + 5
99	,, liver	*M*/1000, *M*/2000, *M*/4000	+20, +14, +6
101	,, ,,	*M*/1000, *M*/2000, *M*/4000	+20, +13, +5
102	,, testis	*M*/1000, *M*/2000, *M*/4000	−16, − 8, −6
104	,, ,,	*M*/1000, *M*/2000, *M*/4000	−25, −15, −6
105	,, ,,	*M*/10,000, *M*/100,000	− 2, 0

The presence of lead ion under these conditions accelerates slightly the respiration of human malignant tissue and also of rat liver. In the latter case, the effect increases with the concentration of lead acetate used. Rat spleen is little affected, while the respiration of rat testis is retarded, the effect increasing with increasing concentration. It is of interest that the effects on normal tissues should vary to this extent.

If the so-called Arndt-Schulz law, previously referred to in connection with the action of lead on glycolysis, is applicable to the case of respiration, it would be concluded as regards malignant tissue that respiration is much less sensitive to the action of lead than is glycolysis. In the case of spleen the effect of lead on both respiration and glycolysis appears to be very small, while with testis the effect is greater and respiration appears to be somewhat more sensitive than glycolysis. These conclusions, as regards relative sensitivity of normal and malignant tissue, are similar to those obtained with mercury.

Application to vital conditions.

It was found by Yabusoe [1926] that the effect of dye-stuffs on tumour glycolysis is much smaller with serum as medium than with Ringer's solution. It would seem probable that the effect of metallic salts at the same ionic concentration would be alike in the two media, since Yabusoe's result is attributed to adsorption of the dyes by serum proteins.

The experiments in which conditions of salt concentration are nearest to those possible *in vivo* are those on the action of lead on glycolysis. If the action of lead could be studied over longer periods of time, such as are available to the organism, it might show itself to be more toxic; the work of Reznikoff [1926] is suggestive in this connection.

Protocols.

Details of several of the experiments referred to previously are given below in order to indicate the degree of accuracy obtained, and the methods of calculation of results which have been used.

Protocol 1.

Exp. 20. *Anaerobic glycolysis of Flexner rat carcinoma.*

Ringer $NaHCO_3 = 0{\cdot}0191\ M$, glucose 0·2 %. $p_H = 7{\cdot}35$.

Gas mixture 5 % CO_2 in N_2.

Vessel	4	5	7	8	10
Cc. Ringer's solution ...	5·0	5·0	5·0	5·0	5·0
Dry weight of tissue ...	3·69 mg.	1·73 mg.	1·52 mg.	2·07 mg.	2·10 mg.
k_{CO_2}	1·078	1·043	1·040	1·009	0·982
Manometer change per 15 mins.:					
0–15′	39·1	16·0	11·4	18·3	20·7
15–30′	35·5	13·8	9·3	15·4	18·6
30–45′	36·2	14·5	11·6	15·4	19·5
$Q_{CO_2}^{N_2}$ (0–45′) ...	43·2	35·6	29·5	31·9	41·3

Protocol 2.

Exp. 57. *Effect of different amounts of $HgCl_2$ on the anaerobic glycolysis of rat spleen in Ringer's solution.*

Vessel	4	5	7	8	10
Cc. Ringer's solution ...	5·0	5·0	5·0	5·0	5·0

After measuring $Q^{N_2}_{CO_2}$ for 30 mins. the following additions of $HgCl_2$ in Ringer's solution were made to the experimental vessels.

Added	—	0·17 cc. *M*/3200 $HgCl_2$	0·17 cc. *M*/800 $HgCl_2$	0·95 cc. *M*/800 $HgCl_2$	—

Rates after addition of $HgCl_2$ expressed as per cent. of initial rates:

Time	Mean of controls 4 and 10	*M*/97,000 $HgCl_2$	*M*/24,000 $HgCl_2$	*M*/5000 $HgCl_2$
10– 40′	101	99	96	89
40–100′	102	95	80	55
100–130′	100	85	79	42
130–160′	100	85	67	38
163–253′	95	76	54	25
253–297′	88	70	43	19
Rates relative to mean of controls = 100:				
25′	—	97	95	100
70′	—	93	78	53
115′	—	85	72	41
145′	—	86	67	37
208′	—	79	57	25
275′	—	79	49	21

The calculated rates obtained from the relation $V = V_0 . e^{-at}$ are given in Table III.

Protocol 3.

Exp. 134. *Effect of HgI_2 in Ringer's solution containing various concentrations of sodium iodide on the anaerobic glycolysis of rat spleen.*

Rates after addition of HgI_2 expressed in per cent. of initial rates (relative to controls = 100) together with the calculated values of a.

Concentration of I′ in Ringer								
M/1000			*M*/100			*M*/10		
t	V	a	t	V	a	t	V	a
15·5	(116)	—	14·5	69·6	1·50	15·5	63·2	1·78
30·5	56	1·13	29·5	45·7	1·59	30·5	31·9	2·25
45·5	39·6	1·21	44·5	29·1	1·67	45·5	22·1	1·99
60·5	28·5	1·24	59·5	14·9	1·92	60·5	7·8	2·53
102	17·6	1·02	101	12·4	1·24	102	4·0	1·89

SUMMARY.

1. Measurements made on the glycolysis and respiration of various tissues agree in the most part with those of other workers. No aerobic glycolysis of liver was found.

2. The toxicity of metallic salts to glycolysis is in the order HgI_2, $HgCl_2 > Cu^{\cdot\cdot} > Zn^{\cdot\cdot} > Cd^{\cdot\cdot} > Ca^{\cdot\cdot}$.

3. Lead ion at low concentrations accelerates slightly the glycolysis of malignant tissues, and at much higher concentrations their respiration. With normal tissues its effects are smaller.

4. The glycolysis of malignant tissue is more sensitive to metallic poisons than that of normal tissues.

5. Glycolysis appears to be more sensitive than respiration to heavy metals in the case of malignant tissue. For normal tissues the converse is true.

6. A mechanism of the action of mercuric halides on glycolysis and respiration is suggested, in which the halide penetrates tissues rapidly, and the inactivation of the enzyme follows a unimolecular law, mercury passing from an adsorption compound to a combination with the enzyme which is inactive.

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XC. THE EFFECTS OF CONGESTION AND HEAT ON THE COMPOSITION OF VENOUS BLOOD SAMPLES TOGETHER WITH SOME OBSERVATIONS ON THE INDIRECT DETERMINATION OF IRON AND CHLORINE IN BLOOD-CORPUSCLES.

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In certain investigations on the blood composition in nephritis it became obvious that the determination of the iron and chlorine content of red blood-corpuscles was beset with errors, many of which would ordinarily pass unnoticed. The effect of congesting and heating the arm on the composition of a venous blood sample taken from the median basilic vein also appears to have been inadequately studied.

The corpuscular iron or chloride may be estimated directly or by calculation from other results.

Now let % Cs = percentage volume of red blood-corpuscles,
W.B.Fe. = whole blood-iron in mg. per 100 cc.
C.B.Fe. = corpuscular blood-iron in mg. per 100 cc.
P.B.C. = plasma blood-chloride in g. per 100 cc.
W.B.C. = whole blood-chloride in g. per 100 cc.
C.B.C. = corpuscular blood-chloride in g. per 100 cc.

The iron is almost entirely contained in the corpuscles and therefore in the absence of haemolysis the following relations hold within experimental error:

$$\text{W.B.Fe.} \times 100 = \text{C.B.Fe.} \times \% \text{ Cs},$$

$$\% \text{ Cs} = 100 \times \frac{\text{W.B.Fe.}}{\text{C.B.Fe.}}.$$

The % Cs in a sample of blood may therefore be calculated if the value of the W.B.Fe. and C.B.Fe. are known.

Now the W.B.C. is the sum of the chloride contained in the corpuscles and in the plasma, therefore

$$100 \text{ W.B.C.} = (100 - \% \text{ Cs}) \text{ P.B.C.} + \% \text{ Cs} \times \text{C.B.C.},$$

and

$$\text{C.B.C.} = \frac{100 \text{ W.B.C.} - (100 - \% \text{ Cs}) \text{ P.B.C.}}{\% \text{ Cs}}.$$

The values of W.B.C. and P.B.C. are readily estimated without appreciable error, so that the value of the C.B.C. obtained by this equation depends on the accuracy of the estimation of % Cs.

The C.B.C. may be obtained in several ways and apart from the chemical analysis its accuracy will depend on the purity of the corpuscular sample.

Separation of corpuscles and plasma is most completely effected by the haemocrit; the reading of % Cs being less than is obtained in a macro-centrifuge even when the haemocrit and centrifuge are spun at the same speed for the same time [Smirk, 1928]. The most accurate estimate of C.B.C. should therefore be obtained by calculation from P.B.C., W.B.C., and the haemocrit reading.

If the reading on a graduated centrifuge tube is used as a measure of the correct % Cs the value of C.B.C. will be too high because the plasma has not been completely removed. If, however, P.B.C. is known, a haemocrit determination on the corpuscular sample may be used to correct C.B.C. obtained by analysis.

In this investigation the blood-iron and blood-chlorides have been estimated by the methods described by Smirk [1927, 1, 2]. The following experiments illustrate the reliability of the chemical methods employed.

Exp. 1. 2 cc. of well-mixed oxygenated blood were delivered to a centrifuge tube and spun for 5 minutes. Exactly 0·5 cc. of plasma was removed in a standardised pipette and used for analysis, the remaining blood being mixed carefully and analysed.

A = original sample of blood.

B = sample of 2 cc. of whole blood from which 0·5 cc. of plasma has been removed.

	Sample A	Sample B
W.B.Fe.	56·0 mg. per 100 cc.	75·0 mg. per 100 cc.
W.B.C.	0·487 g. per 100 cc.	0·430 g. per 100 cc.

P.B.C. on both samples: 0·641 g. per 100 cc.

Calculated value of W.B.Fe. in $B = \frac{56 \times 2}{1{\cdot}5} = 74{\cdot}7$ mg. per 100 cc.

Actual value of W.B.Fe. in B by direct analysis = 75·0 mg. per 100 cc.

Calculated value of W.B.C. in $B = \frac{0{\cdot}487 \times 2 - 0{\cdot}641 \times 0{\cdot}5}{1{\cdot}5} = 0{\cdot}435$ g. per 100 cc.

Actual value of W.B.C. in B by direct analysis = 0·430 g. per 100 cc.

Exp. 2. 3 cc. of well-mixed oxygenated blood were delivered to a centrifuge tube and spun for 5 minutes. Exactly 1 cc. of plasma was removed in a standardised pipette and used for analysis, the remaining blood being mixed and carefully analysed.

C = original sample of blood.

D = 3 cc. of original blood − 1 cc. of plasma.

	Sample C	Sample D
W.B.Fe.	54·9 mg. per 100 cc.	81·8 mg. per 100 cc.
W.B.C.	0·480 g. per 100 cc.	0·402 g. per 100 cc.

P.B.C. on both samples: 0·626 g. per 100 cc.

Calculated value of W.B.Fe. in $D = \frac{54{\cdot}9 \times 3}{2} = 82{\cdot}4$ mg. per 100 cc.

Actual value of W.B.Fe. in D by direct analysis = 81·8 mg. per 100 cc.

Calculated value of W.B.C. in $D = \frac{0{\cdot}480 \times 3 - 0{\cdot}626}{2} = 0{\cdot}407$ g. per 100 cc.

Actual value of W.B.C. in D by direct analysis = 0·402 g. per 100 cc.

It will be seen that there is a close approximation of results by calculation and analysis in both the above experiments.

The following series of observations on a single sample of venous blood illustrates the relationship between the values of C.B.Fe. and C.B.C. and % Cs as determined experimentally and by calculation from the formulae:

$$\% \text{ Cs} = \frac{100 \text{ W.B.Fe.}}{\text{C.B.Fe.}},$$

$$\text{C.B.C.} = \frac{100 \text{ W.B.C.} - (100 - \% \text{ Cs}) \text{ P.B.C.}}{\% \text{ Cs}}.$$

1.	W.B.Fe. by analysis	= 62·6 mg. per 100 cc.
2.	C.B.Fe. by analysis of sample obtained by macro-centrifuging ...	= 121·0 mg. ,,
3.	C.B.Fe. by calculation from (2) and (5)	= 129·5 mg. ,,
4.	C.B.Fe. by calculation from (1) and (6)	= 134·0 mg. ,,
5.	Purity of corpuscular sample obtained by macro-centrifuging as determined by haemocrit	= 93·2 %
6.	% Cs by haemocrit on whole blood	= 46·7 %
7.	% Cs calculated from (1) and (3)	= 48·3 %
8.	P.B.C. by analysis	= 0·647 g. per 100 cc.
9.	W.B.C. by analysis	= 0·459 g. ,,
10.	C.B.C. by analysis of sample obtained by macro-centrifuging ...	= 0·274 g. ,,
11.	C.B.C. by calculation from (7), (8) and (9)	= 0·258 g. ,,
12.	C.B.C. by calculation from (10) and (5)	= 0·247 g. ,,
13.	C.B.C. by calculation from (6), (8) and (9)	= 0·244 g. ,,

It will be observed that the two lowest values of C.B.C. obtained by methods (12) and (13) agree within experimental error. Both depend for their accuracy on haemocrit determinations. The methods (10) and (11) give high results because of admixture of the sample with serum. The % Cs in (7) is too high for a similar reason and the C.B.Fe. in (2) too low. The separation of serum from the corpuscular mass by the haemocrit has not been as complete as the separation in the case of the whole blood sample. In consequence of this, the C.B.Fe. is slightly less in method (3) than in method (4) and the C.B.C. slightly greater in method (12) than in method (13). From these results it follows that the most accurate method is to obtain the corpuscular blood -iron and -chloride by haemocrit observations on whole blood and subsequent calculation from the plasma- and whole blood-chlorides.

The effects of congestion and heat on the composition of venous blood samples.

It is well known, and is illustrated later in this paper, that variations in the CO_2 content of the blood cause movements of chlorine ions between corpuscles and plasma, the total or whole blood-chloride remaining constant.

Any acceleration or retardation of the blood flow may therefore be of importance in investigations of the distribution of chlorine in the blood.

The effect of congestion.

Where veins are normally inconspicuous the tendency when removing blood from them is to leave the tourniquet in position for a longer time until they are more easily visible.

Experiments have been performed on human subjects revealing considerable differences in the composition of venous blood from congested and uncongested veins. The blood in four of these cases was collected in an air-tight syringe, the last bubble of air being replaced by sterile paraffin. The sample was delivered under paraffin to the bottom of a tube containing a small amount of potassium oxalate. The paraffin prevents loss of CO_2 and, by exclusion of air, oxygenation of the sample is avoided. The following results were obtained.

Test	Treatment	W.B.Fe. mg. per 100 cc.	Change in W.B.Fe.	P.B.C. g. per 100 cc.	Change in P.B.C.	W.B.C. g. per 100 cc.	Change in W.B.C.	C.B.C. g. per 100 cc.	Change in C.B.C.
1	None	60·3	+10·7	0·568	−0·006	0·450	−0·010	0·304	+0·035
	Congestion	71·0		0·562		0·440		0·339	
2	None	66·5	+12·3	0·596	−0·005	0·474	−0·014	0·312	+0·005
	Congestion	78·8		0·591		0·460		0·317	
3 *A*	None	64·0	+ 6·7	0·587	−0·008	0·456	−0·008	0·300	+0·017
	Congestion	70·7		0·579		0·448		0·317	
3 *B*	Oxygenation of untreated sample	64·0	+ 6·7	0·612	+0·007	0·456	−0·008	0·253	+0·011
	Oxygenation of congested sample	70·7		0·619		0·448		0·264	
4	None	47·3	+ 0·1	0·568	−0·001	0·472	−0·002	0·301	−0·003
	Slight temporary congestion	47·4		0·567		0·470		0·298	
5 *A*	None	62·6	+ 2·9	0·596	−0·012	0·459	−0·005	0·306	−0·009
	Congestion	65·5		0·584		0·454		0·297	
5 *B*	Oxygenation of untreated sample	62·6	+ 2·9	0·647	−0·010	0·459	−0·005	0·274	−0·005
	Oxygenation of congested sample	65·5		0·637		0·454		0·269	

It will be observed that the whole blood-iron was greater where veins were congested than in uncongested vessels, but unchanged in test 4 where congestion was slight and temporary. The plasma-chloride was diminished and the corpuscular chloride increased wherever the change was definitely outside the range of experimental error. These chloride transferences are due to the greater CO_2 tension of venous blood.

The increase in blood-iron denotes an increase in the proportion of corpuscles and, in consequence of this, there is a fall in the whole blood-chloride, because corpuscles contain less chloride than plasma.

In two experiments, 3 *B* and 5 *B*, the blood was shaken thoroughly with air so that the effect of the increased CO_2 was removed. The considerable increase in the plasma-chlorides and the diminution in the corpuscular chlorides as compared with experiments 3 *A* and 5 *A* are worthy of note.

It is clear, therefore, that when venous samples are used as an index of the chlorine and iron content of the circulating blood it is necessary that the arm should be only temporarily congested and that the tourniquet should be preferably released for several seconds after the needle is in the vein before the blood sample is actually taken. In addition, the venous blood obtained should be thoroughly oxygenated to remove CO_2 and obtain a value of the

plasma-chloride independent of variations in CO_2 produced by local metabolic changes in the arm.

These results are in accord with the observations of Peters, Bulger, Eisenman and Lee [1926], who found an increase in the plasma-proteins and a diminution in plasma-chloride on congesting the source of a venous sample.

The effect of heat.

When the hands and arms are heated by immersion in hot water for several minutes, changes occur in the venous blood composition which, however, are less definite than after congestion. The blood, when withdrawn from the heated arm, appears distinctly arterial. The following results were obtained:

Test	Treatment	W.B.Fe. mg. per 100 cc.	Change in W.B.Fe.	P.B.C. g. per 100 cc.	Changes in P.B.C	W.B.C. g. per 100 cc.	Change W.B.C.	C.B.C. g. per 100 cc.	Change in C.B.C.
1	None	51·5		0·652		0·486		0·285	
	Immersion in hot water	52·2	+0·7	0·661	+0·009	0·485	−0·001	0·277	−0·008
2	None	60·3		0·568		0·450		0·304	
	Immersion in hot water	57·7	−2·6	0·578	+0·010	0·449	−0·001	0·273	−0·031
3	None	66·5		0·596		0·474		0·312	
	Immersion in hot water	74·7	+8·2	0·609	+0·013	0·479	+0·005	0·317	+0·005
4 *A*	None	64·0		0·587		0·456		0·300	
	Immersion in hot water	70·7	+6·7	0·586	−0·001	0·457	+0·001	0·300	0·000
4 *B*	Oxygenation of untreated sample	64·0		0·612		0·456		0·253	
	Immersion in hot water and oxygenation	70·7	+6·7	0·616	+0·004	0·457	+0·001	0·256	+0·003
5	None	47·3		0·568		0·472		0·301	
	Immersion in hot water	44·1	−3·2	0·576	+0·008	0·471	−0·001	0·298	−0·003

In all cases where any chloride transference takes place owing to the diminished CO_2 content of the arterialised venous blood the plasma-chloride is raised.

The corpuscular chloride falls in all cases except 3 and 4 *A*. In the latter no fall in corpuscular chloride would be expected since the plasma-chloride was not increased.

The iron and whole blood-chloride are not uniform in their deviation; the tendency however is for a rise in iron content. The total iron increase in the five tests amounts to 15·6 mg. and the total fall to 5·8 mg. The fact that the whole blood-chloride and iron values do not maintain the theoretical inverse relationship may suggest that the plasma-chloride can interchange with the tissue chlorides in much the same way as with the chloride of the corpuscles. There is, however, no direct evidence of this and changes are of too small an order to allow safe deduction.

SUMMARY.

1. The corpuscular blood-chloride and corpuscular blood-iron have been estimated by direct and several indirect methods. The errors in these determinations have been shown to be due to incomplete separation of plasma from the corpuscles, and not to the chemical analysis.

2. Congestion of the arm causes a concentration of iron and increased venosity in samples from the median basilic vein. This is associated with a reduced percentage of chlorine in the plasma and an increased percentage in the corpuscles, and is of practical importance in blood sampling.

3. Heating the arm renders the venous sample more arterial in appearance and this is associated with a transference of chlorine ions from corpuscles to plasma. The iron content of the blood may be raised.

4. Venous samples should be taken with a minimum of congestion and should be thoroughly shaken with atmospheric or alveolar air if they are to form a reliable index of changes in the plasma-chlorides of the general circulation.

I wish to acknowledge my indebtedness to Prof. H. S. Raper and Dr Craven Moore for their kind interest, criticism and advice, which have alike proved invaluable, and to the Medical Research Council for a grant.

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XCI. THE DETERMINATION OF SUCCINIC ACID IN BLOOD.

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In a recent paper [Clutterbuck, 1927] a polarimetric method was described for the determination of succinic acid in muscle and liver pulp in terms of its conversion into *l*-malic acid and was used for detecting its formation in the pulp, after addition of various substances which had been previously shown to yield succinic acid by oxidation with hydrogen peroxide [Clutterbuck and Raper, 1925, 1926]. No such formation of succinic acid was detected from any of the suggested precursors.

It seemed possible, however, that this result was due either to the inability of the method to detect the production of small quantities of the acid or to the slowness of the series of intermediate reactions, conversion to malic acid, therefore not being attained. The fact that minced organs were used was also regarded as a disadvantage.

It was decided, therefore, to adapt the method of Moyle [1924] for the determination of succinic acid in muscle, by which it was claimed to be possible to estimate very small amounts with an accuracy of $95 \pm 5\ \%$, to its determination in blood with a view to following the succinic acid content of the blood perfusing a surviving liver under various conditions.

In the early experiments, however, the determination by the Moyle method of a known amount of succinic acid present in blood filtrates always gave very low results both when the proteins were removed by acid (mercuric sulphate + sulphuric acid) and by alkaline (zinc sulphate + sodium hydroxide) precipitants. The method, or our way of carrying it out, became, therefore, suspect and it seemed desirable to make a series of careful determinations of standard solutions of succinic acid by direct titration by the silver method and after putting the acid through the whole of the Moyle technique.

The present note deals only with the attempts at determining succinic acid in blood.

Experimental.

Direct titration of succinic acid by the silver method.

When small amounts (5–10 mg.) of succinic acid are precipitated as the silver salt and the silver determined by the Volhard method, the acid is determined with an accuracy of $99 \pm 1\ \%$.

Thus taking 2 cc. of $N/10$ succinic acid solution, very dilute potassium hydroxide was added until just alkaline to litmus, then a drop of very dilute nitric acid to make the solution just acid, then 1 cc. 10 % silver nitrate; finally, the solution was just neutralised with a drop of very dilute ammonium hydroxide. The silver precipitate was allowed to stand for about 4 hours and then transferred to a small Gooch crucible, the filtrate being used several times for washing the precipitate quantitatively on to the filter. The precipitate was then washed by running first 3, then 2, and finally 1 cc. of a very dilute solution of ammonium nitrate from a pipette round the inside of the Gooch crucible. Finally the silver precipitate was dissolved by pouring on to the filter three small amounts of hot dilute nitric acid (1 in 4), cooled and titrated with standard $N/100$ potassium thiocyanate, using iron alum as indicator.

A large number of these determinations have been carried out as controls and the thiocyanate titration has varied from 19·6 to 20·0 cc., indicating that 11·8 mg. of succinic acid is determined with an accuracy of 99 ± 1 %. Using 1 cc. of the standard succinic acid solution, 5·9 mg. of acid is determined with the same accuracy.

The accuracy of the determination of succinic acid using the Moyle technique.

The Moyle technique consists in saturating with ammonium sulphate the solution containing succinic and sulphuric acids, making up to a volume of 20 cc. with saturated ammonium sulphate solution, extracting five times by shaking out with ether, subjecting the extracted material to oxidation to remove lactic acid etc. and then re-extracting with five more portions of ether, the acid being finally determined by the silver method.

A series of experiments was carried out using 5–10 mg. succinic acid, the amount being determined by direct titration as above and used as a control, whilst the same amount was extracted, using five ether extractions under the conditions of the Moyle technique, and then titrated. Finally the same amount was put through the whole of the Moyle technique (2 sets of extraction) and then titrated.

In the early experiments, extraction consisted of the usual 5 minutes' vigorous shaking by hand. Under these conditions, fairly constant but very low results were obtained, the amount of succinic acid obtained after five such extractions being only 90 ± 2 % and after the whole technique 82 ± 2 % of the theoretical. In other words, 1 % had been lost due to titration error, 9 % by the incomplete nature of the first hand extractions and a further 8 % due to loss by the second series of hand extractions.

It was found, however, that part of this error was due to the fact that hand shaking for 5 minutes did not quite attain equilibrium. Ten minutes appeared sufficient but to be quite sure that the whole of the error was not due to this, a number of determinations were carried out, each hand shaking

being timed for 15 minutes. During these determinations it became recognised that in order to get consistent results, great care was necessary to standardise the volume of sulphuric acid and the amount of ammonium sulphate remaining as solid, the volume of ammonium nitrate used for washing the precipitate and the volume of fluid from which the silver salt is precipitated. Under the optimal conditions, however, the amount of succinic acid extracted by five ether extractions was only 92 ± 2 % of the theoretical and by the two sets of extractions as in the Moyle technique, only 86 ± 2 %, so that the loss in the method was 1 % by titration and a further 7 and 6 % respectively in the two series of extractions.

The method as modified and applied to blood.

The standardised Moyle method was then applied to blood with and without addition of known amounts of succinic acid, the blood being deproteinised by means of zinc sulphate-sodium hydroxide and an aliquot portion of the filtrate being used for the determination. The sample of blood used did not contain any acid and the amount of acid recovered (adding about 10 mg.) was 83 ± 3 %. The results with blood were therefore somewhat less consistent than with pure solutions of the acid but the mean value of a number of determinations in blood was only 3 % below that for the corresponding amount in pure aqueous solution.

The ten hand extractions involved in the method proved, however, very laborious and it was therefore attempted to substitute constant ether extraction. Small amounts (10 mg.) of succinic acid were recovered quantitatively by constant ether extraction overnight from saturated ammonium sulphate solution. If in determinations of succinic acid in blood a constant ether extraction was substituted for each set of hand extractions, untrustworthy results were obtained. It was found possible, however, to replace the first series of hand extractions with a constant ether extraction and under these conditions fairly consistent results were obtained of a somewhat greater accuracy than when the two sets of hand extractions were employed, the recovery from blood being 87 ± 3 %.

The method as finally adopted was as follows. 2 cc. (11·8 mg.) of *N*/10 succinic acid solution was added to 10 cc. of a sample of sheep's blood (which did not contain succinic acid) then 78 cc. water, 50 cc. 4·5 % zinc sulphate solution and finally 10 cc. of *N* sodium hydroxide. The proteins were coagulated in a boiling water-bath and the solution was filtered. Generally, 90 cc. of clear filtrate were readily obtained and this volume, 90/150 or 3/5 of the whole, was the usual aliquot taken. This amount of protein filtrate was then evaporated to a small bulk (10 or 20 cc.) cooled, 2 cc. of concentrated sulphuric acid were added, and the liquid was then saturated with ammonium sulphate and transferred to a small constant ether extraction apparatus, the dish being washed out into the extractor with a little saturated ammonium sulphate solution. After extraction overnight, the ether was removed, the

residue was dissolved in about 70 cc. of water, 2 cc. of concentrated sulphuric acid were added and the liquid was heated on the water-bath. Potassium permanganate ($N/10$) was then added until a permanent brown precipitate was obtained and the solution evaporated to about 10 cc. saturated with ammonium sulphate and transferred through the special filter recommended by Moyle to a 100 cc. stoppered measuring cylinder. Successive small amounts of saturated ammonium sulphate solution were then used for washing the dish and filter and were added until the volume reached 19 cc. A little solid ammonium sulphate was then added to the cylinder, the volume being finally 20 cc. Ether (60 cc.) was next added and the cylinder shaken vigorously for 10 minutes, care being taken to prevent loss by the blowing out of the stopper owing to pressure initially generated by the ether. After standing for 10 minutes the ether was siphoned off into a small conical flask containing about 5 cc. of distilled water, and the ether removed. The extraction process was repeated five times and the succinic acid extracted was finally estimated by precipitation of the silver salt and titration with thiocyanate as in the direct titration. In a number of estimations of this type the final titrations varied between 10·1 and 10·8 cc. $N/100$ potassium thiocyanate, and the mean titration of ten results was 10·44. The mean titration corresponds therefore with a titration of $10{\cdot}44 \times 5/3 = 17{\cdot}4$ cc. of thiocyanate, correcting for the aliquot, and the percentage recovery is therefore $\frac{17{\cdot}4}{20} \times 100$ or 87 %.

Summary.

1. The accuracy of Moyle's method for the estimation of succinic acid in tissue has been investigated using a standard solution of the acid. The amount recovered was 86 ± 2 %. This was not as high as that obtained by Moyle in determinations in muscle (95 ± 5 %).

2. The method has been modified and adapted to the determination of succinic acid in blood, the amount of acid recovered being 87 ± 3 %.

In conclusion, the author desires to thank Prof. H. S. Raper for helpful suggestions and criticism.

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XCII. STUDIES ON PECTIN. PART III.

THE DEGREE OF ESTERIFICATION OF PECTIN IN THE JUICE OF THE LEMON.

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During the past few years interest in the chemistry of the pectic substances has revived, and considerable advances have been made in our knowledge. The recent work of Nanji, Paton and Ling [1925] has thrown much light on the constitution of the basal unit; and the conclusions of these workers have been confirmed by Norris and Schryver [1925]. On the other hand very little exact knowledge exists as to the precise nature of these substances *in situ*. Examination after extraction is apt to give results which are misleading, since several changes are known to occur during this process. For example, it is quite certain that de-esterification must occur during the usual processes of extraction to a quite considerable extent. Consequently the methoxyl content of the pectin preparation cannot be taken as that of the pectin as it existed in the plant before extraction.

It is now generally accepted that the pectic substances occur in the plant in at least two forms, one soluble in water, and the other insoluble. Tutin [1923] did not agree with this statement, arguing that the retention of pectin in a form apparently insoluble in water was due solely to mechanical difficulties of extraction. His objections, however, were fully met by the work of Carré [1925], who produced good evidence for the existence of a pectic substance insoluble in water, called by her "pectose," and supposed to be in combination with cellulose. This substance, which will form the subject of a further communication, corresponds to the "protopectin" of other workers. The soluble modification has been supposed by most workers to arise from the insoluble form, although the mechanism of the process is not understood. Further it is clear that the soluble pectin does form a part of the cell wall, not only in young tissue, but in mature plants; in other words it is by no means all of the total soluble pectin which is in solution in the cell sap. Consequently an examination of the condition of the soluble pectin in the cell fluid provides a reliable reflection of the state of the same substance *in situ* in the cell wall.

Von Fellenberg [1918] pointed out that methyl alcohol is a scission product of pectin, and assigned to pectin a formula which contained eight methoxyl groups. He even suggested that pectin might be estimated in plant materials

by the yield of methyl alcohol. The formula given by Nanji, Paton and Ling [1925] includes four galacturonic units, each of which may be present as the methyl ester. Norris and Schryver [1925] conclude that in the case of the soluble pectic substance of the plant, three of the four carboxyl groups are esterified, whilst one is still free to react with a metallic ion in normal salt formation. This conclusion is based on the fact that these workers never found more than 9·2 % methoxyl in any of the many samples of pectin prepared by them in various ways. The usual figure obtained by them was much below 9·2 %. The theoretical percentage for the tetra-methyl ester is 11·76, and that for the tri-methyl ester, 8·94.

More recently Norris [1926] has studied the pectic substances in the juice of the orange, and proved them to be essentially the same as those extracted from the cell wall by purely chemical means. He states that his product from the juice corresponds almost exactly with a tri-methoxylated derivative of pectic acid, having an average methoxyl content of 8·92 %. However, the method of preparation used by Norris is not above criticism, since it probably involves the loss of a portion of the methoxyl content. The oranges which he employed were placed in boiling water for 2 hours to inactivate the enzymes. The hydrogen ion concentration of the juice of oranges is of the order of $p_H = 3{\cdot}5$, and reference to the tables given by Nanji and Norman [1926], and further unpublished work, indicates that heating for 2 hours at this hydrogen ion concentration results in the splitting off of at least 10 % of the total methoxyl content. The effect of natural buffering may be to reduce this somewhat, but nevertheless it is a factor which must not be overlooked.

Experimental.

An investigation was carried out on lemon juice with the same object in view, namely the determination of the degree of esterification of the pectin present in solution in the juice.

About 50 lemons, nearly ripe, were employed in this preparation. The lemons were peeled, cut into two portions and the juice was squeezed out immediately by hand. It was at once filtered through muslin into excess of 95 % alcohol. This precludes any possibility of the intervention of enzyme action, although as a method it is not economical. Ordinary pressing, however, was avoided, as it was not considered desirable for the juice to come in contact with any metallic surface. The precipitated gel, yellowish in colour, was allowed to stand overnight, filtered on fluted filter-papers, and well washed with cold 95 % alcohol. It was then redissolved in cold water, filtered through paper pulp, and reprecipitated by pouring into alcohol as before. This time a greyish-white precipitate resulted. This process of solution and precipitation was repeated five times. The gel was then again dissolved in water, rather more in quantity than before, and the solution filtered through fluted filter-papers before being poured into alcohol. Though very slow, this final

filtration through paper is necessary to ensure that no fibres from the paper pulp are present in the finished product. The gel obtained was then dried with repeated changes of absolute alcohol for over a week, and placed in a vacuum desiccator containing phosphorus pentoxide till of constant weight. The yield from the juice of 50 lemons was rather less than 2 g.

It is to be noted that throughout the preparation the temperature was kept as low as possible, so that de-esterification would not occur. The methoxyl yield was determined by the method described by Nanji and Norman [1926].

The results obtained were as follows:

Ash (i) 11·90 %, (ii) 11·86 %, Mean 11·88 %.

Apparent *methoxyl* yield (on ash-free basis):

(i) 8·64 %, (ii) 8·51 %, Mean 8·57 %.

These results, then, would appear to confirm the conclusions of Norris and Schryver [1925], and of Norris [1926], that the tri-esterified form is the normal state of soluble pectin in the plant. It is possible, however, that this is not the case, since there is no criterion of purity of the pectin preparations. Pectin on precipitation with alcohol carries down with it other substances, which are extremely difficult to remove later even by repeated solution and reprecipitation. This is reflected in the high ash content. Further it is more than possible that there may be present with pectin in the juice some other organic substance, insoluble in alcohol and soluble in water, which will not be removed by the method of purification adopted by Norris [1926], and also used in this work. Accordingly a determination of pectin present in the sample was carried out by the calcium pectate method as modified by Nanji and Norman [1928], and a surprising result was obtained. The calcium pectate yield is the only analytical figure for pectin which is characteristic, and further it has been accepted by many workers as quantitative [cf. Nanji, Paton and Ling, 1925].

The mean yield of calcium pectate from the apparently pure product obtained by the method given above, was only 67·2 %, which on the ash-free product corresponds to 76·3 %. Calculating on this basis then the true methoxyl content of pectin present is (i) 11·56 %, (ii) 11·40 %, giving a mean figure of 11·48 %, the calculated figure for tetra-methoxyl pectic acid being 11·76 %. If this correction for calcium pectate yield is justifiable, and it seems that it is so, it is clear that the soluble pectin as it exists in the juice and in the cell wall is practically completely esterified. It is not claimed that it is uniformly in the tetra-methoxy condition; the acids in the juice would probably prevent this, but the evidence points to a further degree of esterification than that asserted by other workers.

If this correction for calcium pectate yield is applied, the question naturally arises as to the nature of the substance or substances also present with pectin in such preparations to an extent of over 20 %. It was thought at first that a portion might be protein in nature, but the preparation was found to be

free from nitrogen. The furfural figures as recorded by Norris and Schryver [1925], and Norris [1926] for their preparations agree fairly closely with the theoretical figures assuming only pure pectin to be present. The capacity for yielding furfural is not, of course, limited to the pectic substances, so it is clear that the unknown substance must yield approximately the same amount of furfural as pectin, that is, about 20 %, and possess similar solubilities. Norris and Schryver [1925] isolated a hemicellulose associated with pectin in turnips. This, however, was only found to be present to the extent of 3 to 4 %; and further, its yield of furfural was low, being only of the order of 12 %. The substance "hemicellulose C" also described by these workers gives a furfural yield approaching that apparently indicated in this case, but the vague character of these substances renders it impossible to carry the parallel further.

It seems likely that this associated material consists of pectin degradation products, the six-membered pectin ring having been broken, producing compounds of a conjugated galacturonic type retaining in all probability one pentose group. In view of this observation, work is being carried out on the breakdown of pectin beyond the pectic acid stage, and will be described in a further communication.

Summary.

1. Special precautions are taken to obtain a preparation of the pectin from its solution in lemon juice, without the possibility of the occurrence of partial de-esterification.

2. Such a preparation gives an apparent methoxyl content corresponding to a tri-methoxylated derivative of pectic acid.

3. If a correction for the calcium pectate yield of the preparation is employed—the only criterion of purity known—then the corrected figures for methoxyl approach the theoretical value for tetra-methylpectic acid.

4. It is claimed that no good evidence exists for stating that the soluble pectic substance exists uniformly in the tri-methoxylated condition; and that it must be regarded as being almost completely esterified.

5. Some other material, probably pectin degradation products, giving a furfural yield similar to that of pectin, is associated with pectin in such preparations.

In conclusion it is a pleasure to express my thanks to Prof. A. R. Ling for his advice and encouragement.

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XCIII. THE SIGNIFICANCE OF THE NON-FERMENTABLE REDUCING SUBSTANCES OF THE BLOOD IN DIABETES.

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Folin has recently demonstrated that the hyperglycaemia of diabetic blood is not all due to accumulation of glucose. From 15 to 25 % of the sugar in such blood may be represented by other fermentable substances which this author groups under the term of "non-glucose fermentable sugars." The reaction of these sugars to insulin was similar to that of glucose, at least in so far as their concentrations in blood were concerned. These findings renew interest in all the non-glucose reducing substances of blood, very little of which can be accounted for, in the absence of nephritis, by nitrogenous materials.

The greatest difficulties, heretofore, in studying the metabolism of these substances, were due to the technical methods involved in their estimation, since the methods commonly in use for estimating the sugar of human blood measure not glucose alone, but also other reducing substances which are now generally known to be found in blood. Some are fermentable whilst others are not.

The commonest and most logical, theoretically, of older methods of estimating the concentrations of the non-fermentable reducing substances of blood, depended upon measuring the total reducing substances before and after alcoholic fermentation, and with proper precautions it is now possible, with a reasonable degree of accuracy, to estimate the true contents of non-fermentable reducing substances.

Hiller, Linder and Van Slyke [1925] have shown that it is possible to reduce the fermentation period to 20 minutes. At a later date Folin and Svedberg [1926] found that it was possible to reduce still further the fermentation period. More recently Somogyi [1927] has shown that it is unnecessary to incubate blood with yeast even for the brief periods mentioned above. Sugar may be completely removed, at room temperature, in the course of the Folin-Wu precipitation of the blood-proteins, if the water for dilution and laking of the blood is replaced by a 10 % (moist weight) yeast suspension.

In order to study the significance of these non-fermentable reducing substances in diabetes, it was considered advisable, firstly, to obtain standards

by determining, by the technical procedures adopted, the amounts of these substances in normal bloods. Ideas as to these amounts appear to differ. Thus, Hiller, Linder and Van Slyke [1925] found amounts equivalent to 10 to 30 mg. of glucose per 100 cc., whereas Folin and Svedberg [1926] and also Somogyi [1927] found the values to vary within narrower limits. Different procedures were, however, used in each case, either as to the fermentations or blood-sugar determinations.

In this investigation we used Somogyi's method of purifying the yeast and also his method of fermentation, except that for fermentation longer periods and higher temperatures were allowed. For the estimation of the total reducing substances the Folin-Wu blood-sugar method was used. Briefly, the combined technique was as follows.

The material necessary for fermentation was first prepared. A weighed amount of fresh yeast was suspended in about 10 parts of water, centrifuged, and the water decanted. This operation was repeated until the supernatant fluid was clear and colourless, and the last washing gave no greater reduction with the Folin-Wu copper reagent than did a control consisting of distilled water. This procedure removes the adhering particles of wort and other materials found in yeast which contain unfermentable reducing substances. The yeast was then suspended in 10 parts of water and, in order further to avoid errors, 2 cc. of this suspension were also tested for reducing substances by the Folin-Wu process. The reductions found were insignificant. They could not be estimated quantitatively.

As soon as the above process was complete, specimens of blood were obtained. Part of the blood was immediately treated in order to precipitate the proteins and part was fermented as follows.

Into a flask containing 7·5 volumes of a 10 % suspension of yeast prepared as above, was placed 1 volume of blood[1]. After thorough mixing, the flask was allowed to remain in a water-bath at 37° for 7 minutes. One volume of 10 % sodium tungstate was then added followed by 1 volume of 0·66 *N* sulphuric acid. After thorough shaking, the mixture was allowed to stand for 10 minutes. It was then filtered.

The reducing substances of the filtrates obtained before and after fermentation were estimated simultaneously. The necessary precautions for estimating minute amounts of sugar pointed out by Folin and Svedberg [1926] were taken. All values in the tables are expressed in g. glucose per 100 cc. blood.

Values were first obtained, before and after fermentation, from bloods collected in the fasting state. The subjects included 100 non-diabetic adults, 10 non-diabetic children and 100 diabetics, of all ages. A summary of the data will be found in Table I.

By this procedure the amounts of non-fermentable reducing substances were found to vary within narrow limits, namely 16 to 31 mg. per 100 cc.

[1] As Somogyi has shown, it is necessary to allow 7·5 volumes instead of 7 volumes of yeast suspension to correct for the volume occupied by the yeast.

blood. The variations noted were greater than those reported by Somogyi [1927], who used the Shaffer-Hartman method, and approached those found by Hiller, Linder and Van Slyke [1925].

Table I. *Summary of values of non-fermentable reducing substances (g. per 100 cc.) found in fasting bloods of diabetic and non-diabetic subjects.*

Subjects	No. of individuals	Total reducing substances			Range of blood-sugars
		Maximum	Minimum	Average	
Normal adults	100	0·030	0·022	0·025	0·076–0·123
Normal children	10	0·026	0·016	0·023	0·070–0·100
Diabetics	100	0·031	0·024	0·027	0·111–0·333

It was found that, unless determinations were made immediately after collecting the blood, much wider variations were obtained.

Though there were variations between the maximum-minimum values in the large group of individuals, for the individual it appears that the values are remarkably constant. This is shown in Table II. In this table are recorded daily values found in fasting bloods of one normal and two diabetic subjects.

Table II. *Showing daily values of total reducing and of non-fermentable reducing substances (g. per 100 per cc.) obtained in blood of one normal and two diabetic subjects.*

Day	Normal		Diabetic		Diabetic	
	Blood-sugar	N.F.R.S.*	Blood-sugar	N.F.R.S.	Blood-sugar	N.F.R.S.
1	0·080	0·026	0·143	0·018	0·180	0·024
2	0·100	0·022	0·125	0·020	0·166	0·022
3	0·123	0·023	0·151	0·022	0·180	0·026
4	0·075	0·025	0·133	0·019	0·143	0·024
5	0·084	0·026	0·143	0·023	0·181	0·023

* N.F.R.S. = non-fermentable reducing substances.

As has been previously noted by others [Folin and Svedberg, 1926; Somogyi, 1927], the values did not vary with different sugar levels. This will be observed from the data recorded in Tables II and III.

Table III. *Total reducing and non-fermentable reducing substances (g. per 100 cc.) found in bloods of individuals before and after ingestion of 100 g. "commercial" glucose.*

Subject	Substance	Before glucose	30 mins. after	60 mins. after	120 mins. after	180 mins. after
Normal	Total reducing	0·109	0·153	0·185	0·143	0·077
	Non-fermentable	0·028	0·031	0·030	0·030	0·028
Diabetic	Total reducing	0·181	0·357	0·416	0·434	0·454
	Non-fermentable	0·031	0·028	0·029	0·033	0·026
Diabetic	Total reducing	0·270	0·370	0·400	0·476	0·454
	Non-fermentable	0·027	0·024	0·026	0·023	0·023

In Table III will be found the results of three blood-sugar time curves, one obtained in a normal and two in diabetic subjects. It will be seen that, in spite of marked hyperglycaemic bloods, the values of the non-fermentable

reducing substances remained constant for the individual. This is remarkable, in view of the fact that in each of these cases commercial glucose was given. This is well known to be contaminated with both non-glucose fermentable and non-fermentable reducing substances.

The remarkable constancy of the concentrations of these substances in blood, both in non-diabetic and diabetic individuals, in the fasting state and after glucose ingestion, probably explains the fairly reliable results which are obtained with blood-sugar time curves in general, clinically, in the diagnosis of disturbed carbohydrate metabolism.

An interesting observation made by Hiller, Linder and Van Slyke was that by inducing insulin shock in normal rabbits, though the reducing fermentable sugar was reduced to zero, the concentrations of the non-fermentable reducing substances were not altered. This observation appeared to be of sufficient importance to be repeated in the human diabetic. The results of three of such tests are shown in Table IV.

Table IV. *Effect of insulin upon non-fermentable reducing substances (g. per 100 cc.) of bloods of diabetics.*

	Substances	Before insulin	After 1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.
Case 1.	Total reducing	0·333	0·302	0·181	0·062	0·043	Reaction. Glucose given		
	Non-fermentable	0·026	0·023	0·025	0·028	0·027			
Case 2.	Total reducing	0·200	0·133	0·066	0·050	Reaction. Glucose given			
	Non-fermentable	0·018	0·016	0·020	0·018				
Case 3.	Total reducing	0·416	0·333	0·200	0·166	0·080	0·032	Reaction. Glucose given	
	Non-fermentable	0·027	0·028	0·026	0·028	0·025	0·028		

The findings in the human diabetic correspond to those in the normal rabbit. Here, again, are noted, in the fasting state, different values for the different individuals, but remarkably constant values for any one individual throughout the period of observation. The findings in the third subject correspond to those found in the animals by Hiller, Linder and Van Slyke. It will be noted at the end of the observation period that, whereas the total reducing substances correspond to 0·032 g. glucose per 100 cc. the non-fermentable reducing substances correspond to 0·028 g. per 100 cc. Very little significance is attached to the difference, considering the probable error inherent in colorimetric work.

Lawrence [1926] and, later, the writer [1927, 1, 2] have demonstrated the value of determining simultaneously arterial and venous blood-sugars in diabetes. As was originally shown by Foster [1923] in animals, the sugar content rises to a level much higher in the arterial than in the venous blood following glucose ingestion. In diabetic individuals [Lawrence, 1926; Rabinowitch, 1927, 1, 2] the more severe the disease, the less is the difference between the arterial and venous blood values. The difference between these two values is the result of either oxidation or storage of glucose. It would appear that if the metabolism of these non-fermentable reducing substances is disturbed in

diabetes this should be demonstrated in simultaneously determined arterial and venous blood-sugar time curves. In Table V are shown the results obtained in three normal and in three diabetic individuals. It will be noted that, regardless of the differences between the arterial and venous blood-"sugar" values, there were no significant differences noted in the non-fermentable reducing substances.

Table V. *Showing the effect of glucose ingestion upon concentration of total and non-fermentable reducing substances (g. per 100 cc.) of arterial and venous bloods of normal and diabetic subjects.*

			Case 1		Case 2		Case 3	
Subject	Time	Substance	Arterial	Venous	Arterial	Venous	Arterial	Venous
Normal	Before glucose	Total reducing	0·106	0·100	0·084	0·080	0·100	0·100
		Non-fermentable	0·022	0·020	0·018	0·019	0·024	0·026
	After glucose	Total reducing	0·222	0·166	0·196	0·147	0·200	0·153
		Non-fermentable	0·023	0·018	0·021	0·020	0·023	0·022
Diabetic	Before glucose	Total reducing	0·133	0·131	0·111	0·105	0·166	0·164
		Non-fermentable	0·028	0·026	0·020	0·018	0·016	0·015
	After glucose	Total reducing	0·250	0·222	0·250	0·232	0·285	0·263
		Non-fermentable	0·027	0·023	0·022	0·023	0·014	0·018

Summary.

1. An attempt was made to determine whether the non-fermentable reducing substances of blood are of any significance in diabetes.

2. Amounts of non-fermentable reducing substances, by the method employed, were found to vary between 16 and 31 mg. per 100 cc., estimated as glucose. They were also found to be fairly constant for the individual. No significant differences were noted between normal and diabetic bloods.

3. Blood-sugar time curves obtained after the administration of glucose showed no significant differences in the amounts of these substances between normal and diabetic subjects. Arterial and venous blood-sugar time curves, obtained simultaneously, were identical in normal and diabetic subjects, as far as variations of non-fermentable reducing substances were concerned.

4. The amounts of non-fermentable reducing substances were not influenced in the blood of the diabetic by insulin administration.

5. In view of the above findings it is suggested that, in contradistinction to the findings of Folin with non-glucose fermentable sugars, the metabolism of non-fermentable substances is not disturbed in diabetes.

This work was done with the technical assistance of Miss Helen MacGachen and Mr N. H. Goodson.

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XCIV. THE DETERMINATION OF CHLORIDES IN BODY FLUIDS.

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(*Received April 23rd, 1928.*)

In a recent paper [1928] the author showed that the estimation of chlorides in gastric contents could be accomplished by means of a single titration of the Volhard type, without previous removal of protein. The effect of the latter could readily be overcome by introducing a very large proportion of nitric acid, well above that recommended by Whitehorn [1920], for stabilising the end-point of the usual titration.

In continuation of this work, a further step was to ascertain how far the same principle could be applied to other body fluids. Investigation showed that it was capable of general application to all body fluids with the exception of whole blood and corpuscles. The limitation in this case arose from the fact that while the other fluids give with nitric acid a light yellow solution in which the end-point of the Volhard titration can be readily observed, whole blood yields a very dark brown solution which would completely obscure the end-point. A simple decoloration process is therefore a necessary preliminary in this special case.

The problem resolved itself into finding the means of annulling the effect of protein in plasma, for in such a medium that effect would tend to be greatest on account of the high protein concentration, and whatever conditions applied to plasma would almost certainly embrace fluids of lower protein content.

It was found that very slight alteration of the conditions which had been successfully used for gastric contents would in fact achieve the desired end, yielding a new process which reduces the time required for a single estimation to less than half that required even for the neat and rapid method of Smirk [1927]. The present method as finally evolved resembles the latter method in employing a large amount of nitric acid, and follows it in taking advantage of the stabilising influence of acetone upon the end-point. On the other hand, it is wholly dissimilar in principle, in that the essence of the Smirk technique is the vigorous oxidation by ammonium persulphate to destroy protein, a process which in the present method is rendered unnecessary by the choice of appropriate concentration and sequence of reagents.

The end-point is quite sharp and stable. The titration is performed in an almost clear medium, for the silver chloride is largely protected from precipitation by the acid protein, which would also seem to have the effect of stabilising the end-point. Thus, while in a protein-free medium it is desirable to aggregate the silver chloride particles before titration, no disadvantage arises under the present conditions from the fact that the silver chloride is not rendered compact.

For whole blood, decoloration of the solution in nitric acid in order to provide a suitable medium for titration can be readily accomplished in a number of ways, *e.g.* by perhydrol, permanganate etc., but these offer no advantage over the Smirk method of oxidation, and therefore the original technique of this author, with slight variations, has been adopted for this fluid.

Of other biological fluids, attention is drawn to milk, in view of the conclusion but lately drawn by Husband and Godden [1927] expressed thus: "To determine accurately the chloride content of milk volumetrically by precipitation of the chloride as silver chloride, it is essential that the protein be removed before the addition of silver nitrate." The present results show that the conclusion of these authors can only be held to apply to similar conditions of precipitation to those employed in the particular method under their criticism, namely that of Sisson and Dennis [1921].

Methods.

Plasma or serum. Take up 0·1 cc. of plasma or serum in a blood-pipette[1] and discharge into 1 cc. of distilled water contained in a dry boiling-tube or wide test-tube, washing out the pipette by drawing up the water in the usual manner. Add 3 cc. of pure concentrated nitric acid measured from a graduated cylinder of 5 or 10 cc. total capacity, shaking until the protein re-dissolves completely, followed by 0·5 cc. of standard silver nitrate (containing 5·812 g. per litre) delivered from an Ostwald pipette, shaking again during this last addition. Add now 3 to 4 cc. of pure acetone, mix and cool thoroughly. After adding 6 drops (0·3 cc.) of saturated iron alum solution as indicator, titrate from a microburette[1] with alcoholic ammonium thiocyanate of approximately half the strength of the silver nitrate and standardised against the latter. (A convenient solution is made by diluting ordinary aqueous $N/20$ thiocyanate with the required amount—about 2 volumes—of absolute alcohol.) The end-point is reached when one drop imposes a brownish-red tint upon the pale yellow of the original mixture.

Until one has gained complete familiarity with the end-point, it is of great assistance when performing a single estimation to prepare a control tube using the same reagents with 0·1 cc. of any specimen of serum or plasma that is available, except that no thiocyanate is added. When this control is held alongside the tube containing the mixture being titrated, the first departure from the true yellow is readily observed for the marking of the

[1] These pipettes and the microburette must be very accurately calibrated.

end-point. Daylight from a large window immediately behind the operator provides the best conditions of illumination, while the tubes should have white paper immediately behind and below. Table I furnishes some typical comparative results.

Table I.

	Smirk's method	Whitehorn's method	Present method
		(mg. NaCl per 100 cc.)	
A. Plasma: 1.	610	610	609
	612	—	613
2.	585	—	588
	585	—	586
3. After a short dialysis	375	—	378
	380	—	374
B. Serum:	560	—	565
	560	—	560

Whole blood. Save that the same sequence and relative quantities of reagents were preserved as in the application of the present method to plasma, and that no definite weighed quantity of persulphate was used for oxidation, but only a rough knife-point measure, the method applied was essentially that of Smirk.

Puncture fluids such as exudates, transudates, and cyst contents are treated exactly as plasma.

Cerebrospinal fluid (pathological specimens containing excess protein). Take 0·2 cc. of fluid and proceed as for plasma except that 1 cc. of silver nitrate is used.

Milk. Proceed exactly as for plasma, using 0·2 cc. of milk. Typical figures comparing the results so obtained with the method of Austin and Van Slyke [1920] and an adaptation of Smirk's method, are given in Table II.

Table II.

Cow's milk	Austin and Van Slyke's method	Ammonium persulphate oxidation method	Present method
		(mg. NaCl per 100 cc.)	
1	170	168	174
	—	169	170
2	187	187	189
	—	182	186

Urine (pathological specimens with high protein content). Use either 0·1 cc. or 0·2 cc. of urine with 0·5 cc. of $N/10$ silver nitrate and titrate with $N/10$ alcoholic thiocyanate.

Summary.

1. It is shown that the vitiating effect of the presence of protein on the estimation of chloride by the Volhard titration can be readily overcome by use of a high concentration of nitric acid, together with selected proportions and sequence of the reagents normally employed.

2. Use is made of this fact to devise simple and ready methods of estimating the chloride content of various body fluids without the previous removal of protein.

3. The method is not directly applicable to those fluids which, like whole blood, give rise to a dark solution in nitric acid.

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XCV. THE SPECIFICITY OF ERGOSTEROL AS PARENT SUBSTANCE OF VITAMIN D.

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The study of the antirachitic activity induced in various sterols by irradiation, which led to the discovery of ergosterol as the most potent source of vitamin D [Rosenheim and Webster, 1926; 1927, 1], has subsequently brought to light an interesting relationship between chemical structure and potential biological activity.

The most characteristic general features of the sterol structure are the presence in the molecule of (1) a hydroxyl group, (2) one or more unsaturated carbon linkages, and (3) a system of four reduced rings. An analysis of these molecular structures in their relation to induced antirachitic potency has so far failed to clarify the position with regard to the function of the hydroxyl group. We have shown [Rosenheim and Webster, 1927, 2] that two esters of ergosterol, the acetate and the benzoate, are rendered as highly antirachitic as ergosterol itself by irradiation. This activation may, however, be feasibly preceded by a photochemical hydrolysis, and since the removal or replacement of the hydroxyl group in ergosterol cannot be effected without destruction of the molecule [Reindel, Walter and Rauch, 1927] the bearing of the OH group on the vitamin formation cannot at present be experimentally studied from this direction.

On the other hand, it has become evident that not only the presence of the unsaturated carbon linkages, but also their number, is a deciding factor in the photochemical production of vitamin D. For it was found that the naturally occurring saturated sterols, coprosterol and α-amyrol, as well as the artificially reduced sterols, dihydrocholesterol and dihydrositosterol, remain inactive after irradiation. Further, neither cholesterol nor sitosterol[1], with one double bond, nor stigmasterol, cholesterylene and oxycholesterylene,

[1] The activity of irradiated α-sitosterol [Hess and Anderson, 1927], when administered in large doses, seems to have been due to an admixture of ergosterol, or a similar labile sterol. Since α-sitosterol cannot be purified by bromination, the removal of ergosterol from this admittedly non-homogeneous substance is not easily effected. The slight activity after irradiation observed in some specimens of cholesterol, purified by the bromine treatment [Jendrassik and Keményffi, 1927; Bills, Honeywell and MacNair, 1928], would seem to show that even this treatment may fail occasionally to remove the last traces of ergosterol. This is evident by the latter observers' spectroscopic findings, which show the absorption bands of ergosterol besides those due to cholesterylene [see also Heilbron, Morton and Sexton, 1928].

with two such linkages, can be activated. On the other hand ergosterol, with three such linkages, possesses this property to a high degree.

It became therefore of interest to investigate, in the first instance, whether the photochemical change connected with vitamin formation was specific for ergosterol, or whether other substances with three or more double bonds, but not possessing the sterol ring-structure, would also yield the vitamin on irradiation. We had already previously established that squalene, $C_{30}H_{50}$, the open-chain dihydrotriterpene with six double linkages occurring in the livers of elasmobranchs, cannot be activated and the same holds for nerolidol and *pseudo*-ionone, olefinic oxygenated terpenes with three double bonds [Windaus and Holtz, 1927]. In this connection we also investigated the behaviour on irradiation of the unsaturated open-chain amino-alcohol sphingosine, $C_{17}H_{35}NO_2$, a cleavage product of the cerebrosides. According to Zellner and his collaborators, similar nitrogenous substances, the cerebrins, constantly occur in fungi together with ergosterol, from which they can only be separated with difficulty [Rosenthal, 1922]. Neither the cerebroside phrenosin, to which antirachitic properties had been ascribed by Stepp and Woenckhaus [1926], nor sphingosine itself possessed, however, any antirachitic action either before or after irradiation. Accepting from these negative results the tentative conclusion that unsaturated open-chain compounds cannot be activated, there remains the possibility that isomers of ergosterol, differing from it in the relative position of the three double linkages, might be activable.

iso-Ergosterol is obtained from ergosterol by a relatively mild chemical treatment, that is, removal of hydrochloric acid from its hydrochloride [Reindel, Walter and Rauch, 1927]. Through the kindness of Prof. Windaus, we were enabled to investigate Reindel's own preparation. We found it inactive both before and after irradiation, and our results are in agreement with those of Windaus and Holtz [1927]. Another isomer of ergosterol, *neo*-ergosterol, has recently been obtained by distillation of ergopinacol and has also failed to be activated by irradiation [Windaus and Borgeaud, 1928]. Zymosterol, the only naturally occurring sterol of this class, which accompanies ergosterol in yeast [Smedley-MacLean, 1928], has not yet been biologically examined. In distinction from ergosterol, however, the new sterol possesses no selective absorption in the ultra-violet, and, in view of the correlation existing between antirachitic activity and ultra-violet absorption, it would be surprising if zymosterol, when completely freed from ergosterol, acquired antirachitic activity on irradiation[1].

The position of ergosterol as parent substance of vitamin D would, therefore, appear to be unique, except for the statement of Windaus [1927] that

[1] Hume, Smith and Smedley-MacLean [1928] have since established that irradiated zymosterol has no antirachitic properties. We have recently succeeded in isolating a sterol, associated with ergosterol in ergot (Tanret's fungisterol?) and found that it resembles zymosterol in not possessing any selective ultra-violet absorption. Preliminary experiments indicate that it also cannot be rendered antirachitic on irradiation (June 4th, 1928).

digitaligenin, $C_{23}H_{30}O_3$, a substance structurally related to cholic acid, and therefore to the sterols, could also be activated by irradiation [see also Windaus and Holtz, 1927]. Since digitaligenin, the anhydro-aglucone of the digitalis glucoside *digitalinum verum*, is a hydroxy-lactone containing three double linkages, its activation would not seem improbable. A statement coming from these authors deserves serious attention and would obviously be, if confirmed, of considerable theoretical importance. It has already been accepted in the literature and has led to the deduction of the existence of more than one vitamin D in nature.

A detailed consideration of Windaus and Holtz's experimental technique, however, leaves their conclusions open to doubt and we found on a critical repetition of their experiments that digitaligenin, irradiated under the usual conditions, was entirely devoid of antirachitic properties.

This fact, together with the evidence already available, strengthens the assumption that not only a typical ring-structure but also the specific relative positions in which the three double bonds are present in the molecule of ergosterol are essential for the photochemical conversion of a sterol into vitamin D.

Experimental.

The animal experiments were carried out by our usual technique. Except in the case of some of the earlier experiments, in which a thin film of the substance was irradiated, ethereal or alcoholic solutions of the substances were exposed to the radiations of a mercury vapour lamp (atmospheric type). After removal of the solvent in a current of nitrogen, the residue was dissolved in liquid paraffin or in inactive olive oil and a suitable dose administered separately to each rat. In every case control experiments with the non-irradiated substance gave negative results.

Sphingosine was prepared by hydrolysis of pure phrenosin [Rosenheim, 1916]. A thin film of the substance was irradiated for 30 minutes and dissolved in liquid paraffin. Daily doses of 2 and 4 mg. neither prevented nor cured rickets.

Oxycholesterylene, obtained by oxidation of cholesteryl acetate with chromic acid [Mauthner and Suida, 1896], was equally inactive under the same conditions in doses of 0·5 mg.

iso-*Ergosterol* [Reindel, Walter and Rauch, 1927] was tested in the same way in doses of 0·5 mg. and 1/1000 mg. and found to be devoid of antirachitic activity. The two last substances were examined with the same result by Windaus and Holtz [1927], who irradiated them in liquid paraffin solution instead of in a thin film. It is suggestive, in view of the criticism of their technique (see later), that the only positive result recorded by Windaus and Holtz with a substance other than ergosterol (or its acetate), namely, digitaligenin, was obtained by irradiation in olive oil as a solvent.

Digitaligenin was prepared by hydrolysis from *digitalinum verum* according to Kiliani [1914] and had the melting point 212°, as stated for the pure substance by Windaus and Bandte [1923]. We are greatly indebted to our friend, Dr M. Guggenheim, Basle, through whose offices a supply of this valuable substance was kindly put at our disposal by Messrs Hoffmann, La Roche, Basle.

(*a*) *Biological evidence.*

The lowest active dose of irradiated digitaligenin had not been determined by Windaus and Holtz, who stated that 1/100 and 1/200 mg. were active. We therefore investigated its action in smaller as well as in larger doses. The substance was irradiated in 0·01 and 0·2 % alcoholic solution, in a quartz cell of 10 mm. thickness, for periods, varying in different experiments, of 30 minutes, 1 hour and 2 hours. One animal in each set of experiments, in which rats of the same litter were employed, received a daily dose of 1/10,000 mg. of irradiated ergosterol, prepared under conditions similar to those employed in irradiating digitaligenin. The results need not be given in detail since all the doses, 1/10,000, 1/5000, 1/250, 1/100 up to 1 mg., of irradiated digitaligenin gave entirely negative results, whilst the rats receiving 1/10,000 mg. of irradiated ergosterol were free from rickets. It follows from these results that irradiated digitaligenin, even when given in doses 10,000 times larger than those which are effective in the case of irradiated ergosterol, is devoid of antirachitic activity.

(*b*) *Spectroscopic evidence.*

The above conclusion is in harmony with the evidence obtained by the spectroscopic examination of digitaligenin. We found, as was to be expected in the case of a highly unsaturated substance, that digitaligenin showed in alcoholic solution an intense absorption in the ultra-violet, differing entirely in character and position, however, from that of ergosterol. Instead of the three well-defined absorption bands of ergosterol, of which that at 280 $\mu\mu$ is the most intense and characteristic, digitaligenin shows only one band with a maximum at 340 $\mu\mu$, extending from 270 $\mu\mu$ to 390 $\mu\mu$. In addition, there is general absorption, and possibly secondary bands, in the region below 250 $\mu\mu$. Moreover, the broad absorption band of digitaligenin remains practically unchanged after 2 hours' irradiation, whereas the absorption spectrum of ergosterol rapidly changes under these conditions.

It may be suggested that the antirachitic action of irradiated digitaligenin, reported by Windaus and Holtz, was due to the fact that the actual irradiation of the substance was carried out on a 0·01 % solution in olive oil (see above). The obvious criticism arises that the choice of olive oil as a solvent for this type of experiment is an unsuitable one. It is an established fact, which was recognised early in the history of the subject [Steenbock and Black,

1925], that olive oil itself, in common with other vegetable oils, acquires antirachitic properties on irradiation. Further, Windaus and Holtz obtained their positive results in experiments in which relatively large doses (100 mg.) of irradiated olive oil were administered daily when testing the action of digitaligenin. We found that much smaller doses (40–50 mg.) of such oils, three different specimens of which were tested[1], completely prevented rickets in every case. In the single negative control experiment with irradiated olive oil, mentioned by the above authors, the oil was subjected to the intense irradiation of the magnesium spark for 3 hours. Since it is well known that prolonged irradiation destroys the antirachitic activity of vegetable oils and vitamin D, it may be suggested that, for such a control experiment to be convincing, a substance of equally powerful protective absorption in the ultraviolet as digitaligenin should have been added to the olive oil.

SUMMARY.

Evidence is presented which strengthens the assumption that only a molecular structure such as that possessed by ergosterol enables a sterol to be photochemically converted into vitamin D, and confirms the evidence already available for the view that ergosterol is the specific parent substance of vitamin D.

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[1] One of these specimens was kindly procured for us as guaranteed pure "virgin oil" directly from the grower by Mr L. Erles, in Malaga, Spain.

XCVI. STRUCTURE AND ENZYME REACTIONS. PART IV.

THE SYSTEM GLYCOGEN-AMYLASE-LIPOIDS.

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In the preceding parts of this series [Przylecki and co-workers, 1927, 1928] it was shown that the introduction of an adsorbent into a system in which either the enzyme, the substrate or the products of reaction undergo adsorption may profoundly influence the velocity of the enzymic reaction. The results obtained were applied to certain biological problems as an explanation of the mechanism whereby the structure of a cell may influence reactions.

In Part I of this series it was established that for the system urea-urease-charcoal, in which only the enzyme underwent adsorption, no retardation of the enzymic decomposition of urea was brought about by the presence of this adsorbent. Part II dealing with the system polysaccharide-amylase-charcoal, exhibited a different behaviour. Here both the enzyme and the substrate undergo adsorption, that of the latter being reversible in the presence of an appropriate concentration of narcotics. The adsorbed enzyme retained its amylolytic activity, so that it was able to decompose glycogen in solution with its usual efficiency. Where, however, the enzyme and the substrate were simultaneously adsorbed, considerable retardation of hydrolysis took place. Finally, in Part III, it was shown that the introduction of coagulated egg-albumin instead of charcoal yields parallel results.

In this paper, the same problem will be considered, with the use of a different cell-constituent, namely the ether-soluble substances (lipoids), as the adsorbent.

The theoretical aspects of this question have been fully dealt with in the previous parts of this series, to which those interested should refer.

Experimental.

The experimental programme consisted in elucidating the following points: adsorption of enzyme and of substrate on lipoids, fats, etc., their elution, and the effect of the presence of the adsorbent upon the kinetics of the reaction.

The adsorbent consisted of an intimate mixture in equal proportions of cholesterol, lecithin, palmitic acid and calcium glycerophosphate, this mixture being referred to under the general term of lipoids. The amylase used was purchased from Merck in powder form and the substrate taken was glycogen.

1. *Adsorption and elution of amylase.*

A series of flasks containing 23 cc. of buffer solution, p_H 6·5, and 2 cc. of 0·5 % amylase solution containing 1 % of sodium chloride together with, in certain cases, 2 g. of the above mixture, and the same with 0·5 *M* propyl or 0·07 *M* butyl alcohol solution, were placed for 3 hours in a mechanical shaker and the contents of the flasks then filtered. To 20 cc. of each filtrate 10 cc. of 1 % glycogen was added, and the flask kept for 4 hours at 37°, after which the reducing sugar content was determined by Benedict's method. That of Bertrand was not here practicable, as the filtrate when rendered alkaline became unfilterable. The results, given in Table I, indicate that amylase is under these conditions adsorbed to an extent of 70 %, and that the addition of propyl alcohol up to 0·5 *M* or of butyl alcohol up to 0·07 *M* solution leads to an almost quantitative elution of the enzyme, *i.e.* only 5 % remains in adsorption.

Table I. *Adsorption and elution of amylase.*

Mg. glucose in 30 cc. solution, using 1 % glycogen and 2 g. of lipoids.

		With adsorbent					
No. of exps.	Without adsorbent	No alcohol	% adsorption	0·07 *M* butyl alcohol	% adsorption	0·5 *M* propyl alcohol	% adsorption
2	53·8	16·2	67	50·7	6	52·0	4

2. *Adsorption of glycogen.*

A series of flasks containing 25 cc. of 1 or 0·5 % glycogen were shaken for 3 hours with and without the addition of lipoids and alcohols, filtered, and a measured volume of the filtrate was diluted to 30 cc. and hydrolysed by heating for 4 hours at 100° with 1·5 cc. of concentrated hydrochloric acid, after which the acid was neutralised by the addition of excess sodium carbonate and reducing sugars determined. The results (Table II) indicate that glycogen is not adsorbed upon these lipoids.

Table II. *Adsorption of glycogen.*

Mg. glucose in 5 cc. of filtrate, using 0·5 and 1 % glycogen and 2 g. of lipoids.

			With adsorbent			
Glycogen %	No. of exps.	Without adsorbent	No alcohol	0·07 *M* butyl alcohol	0·5 *M* propyl alcohol	% adsorption
1	2	19·1	20·62	19·27	20·55	0
1	2	20·62	21·22	—	—	0
0·5	2	9·46	9·79	9·14	10·13	0
0·5	2	10·25	10·34	—	—	0

3. *Elution of adsorbed ferment by glycogen or water.*

A number of experiments were necessary for the elucidation of this matter. In determining the elution of adsorbed amylase by glycogen, advantage was taken of the fact that this enzyme has practically no action at 0°. A series of flasks containing buffer solution and amylase, with or without lipoids, was shaken as before for 3 hours, after which some were filtered immediately, whilst others were cooled to 0°. An equal volume of 2 % glycogen solution at 0° was then added, and the flasks were shaken at 0° for 3 hours, after which the contents were filtered in an ice-chest, and placed in a thermostat at 37° for 4 hours, together with those flasks whose contents had been filtered immediately and to which an equal volume of 2 % glycogen solution had been added subsequently. The results, given in Table III, show that a certain amount of elution has taken place.

Table III. *Elution of amylase due to glycogen and water.*

Mg. glucose in 10 cc. of filtrate, using 2 % glycogen solution.

		With adsorbent					
No. of exps.	Without adsorbent	Glycogen added before filtering	% hydrolysis	Water added before filtering	% hydrolysis	Glycogen and water added after filtering	% hydrolysis
2	14·45	7·2	50	5·1	35	4·8	33
2	21·7	10·2	47	—	—	7·9	36
2	17·6	8·7	49	—	—	—	—

In order to verify whether this is due to the specific elutive action of glycogen, or whether it is merely due to dilution, an additional experiment was performed.

25 cc. of a 0·04 % solution of amylase, p_H 6·5, was shaken with 3 g. of lipoid mixture for 3 hours, cooled to 0°, 25 cc. of 2 % glycogen solution at 0° were added, and the whole was shaken for a further 3 hours at 0°, after which the mixture was filtered at 0°, half the volume of the filtrate of water added, and the whole kept at 37° for 4 hours. A second pair of flasks was put through the same procedure, except that the order in which the glycogen and water were added was reversed. A third pair of solutions was filtered immediately after shaking and a double volume of 1 % glycogen added to the filtrate, and finally a fourth pair underwent the same manipulations as the first pair, except that lipoids were absent. The glucose content of all the solutions was then determined. The results of this experiment, given in Table III, show that where glycogen solution is added immediately, the amount of amylase in the filtrate is 50 % of that in the absence of adsorbent, where water is added first, 35 % and, where the amylase solution is filtered immediately, 33 %. It would hence appear that glycogen has a considerable elutive influence not possessed by water.

The dilution by water is here two-fold, and in order further to establish this point four-fold dilution was next employed. A pair of flasks containing

25 cc. of a 0·04 % amylase solution was as before shaken 3 hours with 3 g. of lipoid mixture, filtered, 30 cc. of buffer solution were added to 10 cc. of the filtrate, and 40 cc. of 2 % glycogen solution. A second pair of flasks containing 100 cc. of buffer solution, with the same quantity of amylase as the first pair, was shaken and filtered, an equal volume of 2 % glycogen being added to the filtrate; a third pair containing the same quantity of solution but without lipoid mixture was put through the same manipulations. After keeping at 37° for 4 hours, the reducing sugar content was determined. The results given in Table IV indicate that even a four-fold dilution of amylase solution provokes no perceptible elution.

Table IV. *Effect of dilution. Mg. glucose found in* 20 *cc. filtrate using* 2 % *glycogen.*

		With adsorbent	
No. of expe.	Without adsorbent	Diluted before shaking	Diluted after shaking
2	7·35	4·2	4·0

4. *Kinetics.*

A number of flasks containing 25 cc. of 0·04 % amylase solution were shaken 3 hours with 3 g. of lipoid mixture, and 25 cc. of glycogen solution, either 1 or 2 %, added. The same was repeated with a second series of flasks, introducing butyl or propyl alcohol up to 0·07 and 0·5 *M* respectively, and to a third series the same volumes of solutions but no lipoids were added. All flasks were then left for 4 hours in a thermostat at 37°, after which they were filtered, and reducing sugars determined. The results (Table V) show that for 2 % glycogen the hydrolysis is only 40·4 to 49·8 % of that in the absence of an adsorbent, and for 1 % glycogen 61·2 to 75·6 %. The addition of butyl alcohol to the 1 and 2 % glycogen reaction mixtures raises hydrolysis to from 91·8 to 98·0 % of that in the absence of an adsorbent, whilst the action of propyl alcohol is less marked.

Table V. *Influence of adsorbent upon the kinetics of the hydrolysis of glycogen by amylase. Mg. glucose in* 10 *cc. filtrate.*

			With adsorbent					
Glycogen added %	No. of exps.	Without adsorbent	No alcohol	% hydrolysis	0·07 *M* butyl alcohol	% hydrolysis	0·5 *M* propyl alcohol	% hydrolysis
2	2	16·7	8·4	49·8	15·5	92·7	13·2	79·8
2	2	17·8	7·2	40·4	17·6	98·0	7·8	43·8
1	2	11·1	8·4	75·6	—	—	10·1	91·0
1	2	9·8	6·0	61·2	9·0	91·8	6·6	67·3

In view of the possibility that retardation of reaction is due to mechanical causes, *i.e.*, that the adsorbed enzyme, whilst remaining active, reacts more slowly as a result of a decline in the number of collisions between the molecules and those of the substrate, a similar experiment was carried out, with the

difference that the reaction flasks were shaken whilst in the thermostat. The results (see Table VI) indicate that under these conditions shaking has no influence upon the velocity of reaction.

Table VI. *Influence of shaking upon velocity of enzymic hydrolysis.*

Mg. glucose found in 10 *cc. filtrate, using* 1 % *glycogen solution.*

No. of exps.	No lipoids	Lipoids with shaking	Lipoids without shaking
2	10·3	6·6	6·6

Discussion.

The above experiments show that amylase undergoes a considerable degree of adsorption upon the mixture of ether-soluble substances used. The adsorbed amylase can, however, be eluted almost quantitatively by the addition of an appropriate quantity of propyl or butyl alcohol; in this respect lipoids differ from protein, as also in the partial elution produced by the action of glycogen. Again, lipoids do not adsorb glycogen, in contrast to the adsorbents studied previously.

The system glycogen-amylase-lipoids would in the above respects appear to be similar to the systems urea-urease-charcoal and glycogen-amylase-protein (alcohol) described in Parts I and III, in so far as only the enzyme is in adsorption, the substrate being practically unadsorbed. In these cases, little retardation of reaction was observed, pointing to the unimpaired activity of the enzyme in spite of adsorption. Here, however, reaction is retarded to an extent commensurate with that of the quantity of enzyme in adsorption, pointing to the very strong probability of amylase losing its activity on adsorption upon lipoids.

This retardation cannot be due to purely mechanical causes, since shaking in no way affects the velocity of hydrolysis in the presence of an adsorbent.

The elution of amylase from adsorption on lipoids by glycogen is of interest, inasmuch as most elutives act as a result of their preferential adsorption. Here, however, glycogen is not adsorbed, so that the elution must be due to some different cause. This is possibly the decline in the concentration of free amylase in solution as a result of its combination with the substrate.

For the sake of clarity, the different properties of charcoal, protein and lipoids as adsorbents are tabulated in Table VII.

Table VII.

		Adsorption of		Elution of Enzyme by		Elution of Substrate by	Activity of adsorbed enzyme
Adsorbent	Substrate	Enzyme	Substrate	Alcohols	Substrate		
Charcoal	Urea	+	−	−	−	−	+
Charcoal / Protein	Glycogen	+	+	−	−	+	+
Lipoids	„	+	−	+	+	−	−

The behaviour of these two cell constituents, protein and lipoids, is in almost every case contrasting, and points to the great complexity of the mechanism of the processes taking place within the cell, which possesses the means of modifying enzymic reactions in so manifold a manner.

Research is now in hand at this laboratory upon the effect of applying simultaneously both protein and lipoids. In the absence of alcohols retardation of reaction should be observed. The effect of the addition of narcotics should, where protein is present in excess, be to accelerate hydrolysis as a result of elution of substrate; whether the amylase eluted from lipoids is immediately adsorbed upon protein, or whether an enzyme-substrate compound, adsorbed on protein only to a small extent, is formed will be shown by experiment. In all probability, however, the behaviour of such systems will resemble that of protein systems alone, except where lipoids are present in excess. Within the cell, locational influences may further come into play whereby an enzyme eluted from lipoids situated in one part of a cell need not necessarily undergo adsorption upon protein situated in a different part of the cell. Thus the difference between the livers of summer and of winter frogs, mentioned by Lesser [1924] and by Przylecki, and consisting in the almost complete absence of glycogenolysis during the winter months, might be explained not only by the simultaneous adsorption of amylase and glycogen upon protein, but possibly also by the exclusive adsorption of the enzyme upon lipoids.

It further appears probable that Lesser's [1927] observation, that alcohols cause augmented secretion of amylase from the submaxillary glands, can be explained on the assumption that it is adsorbed upon lipoid constituents of these glands.

SUMMARY.

1. Amylase is adsorbed upon lipoids to about 67 % of the quantity in solution.

2. Adsorbed amylase is quantitatively eluted by narcotics, partly by glycogen and hardly at all by water.

3. Glycogen does not undergo adsorption upon lipoids.

4. The addition of lipoids to the system glycogen-amylase causes retardation of hydrolysis.

5. Amylase adsorbed upon lipoids is thereby inactivated; its activity can be restored by elution.

6. The enhanced secretion of amylase from the submaxillary glands under the influence of alcohol observed by Lesser is perhaps explicable on the basis of the above findings.

This paper was, similarly to the rest of the series, undertaken at the suggestion and under the supervision of Prof. St J. Przylecki, to whom I wish to express my sincere gratitude for his co-operation.

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XCVII. THE DISAPPEARANCE OF INTRAVENOUSLY INJECTED α-, β- AND αβ-GLUCOSE FROM THE BLOOD.

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(*Received April 25th, 1928.*)

The effect of the intravenous injection of α-, β- and αβ-glucose on the blood-sugar has been studied by Lipmann and Planelles [1924] and Thannhauser and Jenke [1924].

As the results of these workers did not lead to conclusive or identical results, it seemed desirable to repeat the work. The work described in this note was carried out on rabbits, as in the experiments of Lipmann and Planelles.

Lipmann and Planelles found that α- and β-glucose were removed from the blood with equal ease, but that αβ-glucose caused a greater elevation of blood-sugar and a slower return to normal.

Thannhauser and Jenke, on the other hand, who studied the problem on normal and diabetic human subjects, found that β-glucose caused a slightly greater increase of blood-sugar and a slower return to normal than αβ-glucose: that the sugar in the urine was greater after β- than αβ-glucose: that ketone excretion in the diabetic was more diminished after αβ- than β-glucose. From this they conclude that α-glucose is more easily metabolised than β-glucose.

The results described in this paper give the impression that the difference, if any, in the utilisation of the α- and β-forms of glucose by the mammalian organism is not shown by this method of experiment.

The average rise of blood-sugar at 15, 30, 45 and 60 minutes after the injection (values obtained where necessary by interpolation from the observed values) is slightly greater after α-glucose than after the other two forms, but the differences are insignificant, and if the experiments 2, 5 and 6 are omitted the results for the three sugars are nearly identical (Table I). The three experiments the omission of which is suggested were early in the series, when the dose was rather greater than was used later in the work (nearly 1 g. per kg., instead of 0·5 g.).

When the three curves are obtained from the same animal, as rabbits (3), (6) and (7), the results are not identical, but they are not more different than would be expected even in successive curves using the same sugar.

Table I. *Average rise in blood-sugar above the fasting value, in mg. per* 100 *cc. blood.*

Including all experiments				Excluding Exps. 2, 5 and 6			
	Rise in blood-sugar after				Rise in blood-sugar after		
Time	α-	αβ-	β-	Time	α-	αβ-	β-
15′	118	90	96	15′	94	91	96
30′	73	51	53	30′	63	56	53
45′	49	31	23	45′	34	32	23
60′	22	8	19	60′	5	4	19

If we examine the results of Thannhauser and Jenke carefully, it will be seen that there is really very little difference between the β- and αβ-forms. The difference in the blood-sugar curves is easily accounted for, for example, by slight differences in the rate of injection of the large amount of solution needed (500 cc.), while the differences in the ketone and sugar excretions in the diabetics are within the range of expected variations.

The results of Lipmann and Planelles are rather more difficult to explain. Though not specifically stated in their protocols, it would seem that 1·4 g. glucose in the form of a 7 % solution in normal saline was used, the volume of fluid injected being 20 cc. With two exceptions, Tables II and IV, No. 39, the blood-sugar continues to rise for many minutes after the injection. In the experiments described in this paper this was found to occur only when some of the solution was accidentally injected into the tissues outside the vein. If the injection was completely intravenous, the blood-sugar was found to fall continuously from the first determination. An example of the sort of figures obtained when the injection is not wholly intravenous is given here.

Exp. 19. *Some of the solution injected outside the vein.*

Rabbit 1, 1720 g. 1 g. β-glucose.

Time	Blood-sugar g. per 100 cc.
Before injection	0·117
11 mins. after	0·206
25 ,,	0·204
47 ,,	0·180
80 ,,	0·142
112 ,,	0·131

The slow fall in this case contrasts markedly with the rapid fall from the beginning in the experiments where the injection is wholly intravenous.

Experimental.

The α- and β-glucose were prepared by the recrystallisation of ordinary glucose from acetic acid under the conditions described by Hudson and Dale [1917]. The purity of the specimens used was checked by their having the correct rotatory power.

The rabbits were not fed from the afternoon preceding the day of experiment, *i.e.* usually for a period of about 16 hours.

Protocols of experiments.

(The figures for the blood-sugar are g. per 100 cc.)

αβ-Glucose.

Rabbit No.:	1	4	3	6	7
Exp. No.:	2	4	10	21	22 *a*
αβ-Glucose:	1·5 g.	1·5 g.	1·5 g.	1 g.	1·2 g.

Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar
Before	0·102	Before	0·108	Before	0·105	Before	0·089	Before	0·102
5	0·204	5	0·238	5	0·234	7	0·192	7	0·215
20	0·175	9	0·211	16	0·204	30	0·133	30	0·149
40	0·130	14	0·204	31	0·176	45	0·107	59	0·111
70	0·119	27	0·176	61	0·108	60	0·078	75	0·105
100	0·121	44	0·155	95	0·082	135	0·091	105	0·103
—	—	76	0·093	—	—	—	—	—	—
—	—	130	0·093	—	—	—	—	—	—

α-Glucose.

Rabbit No.:	1	2	4	4	6	7
Exp. No.:	5	6	8	11	22	23 *a*
α-Glucose:	1·5 g.	1·5 g.	1·5 g.	1·5 g.	1 g.	1·2 g.

Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar
Before	0·089	Before	0·076	Before	0·085	Before	0·076	Before	0·091	Before	0·114
6	0·277	6	0·217	5	0·238	5	0·183	8	0·192	7	0·244
13	0·243	14	0·205	16	0·200	15	0·142	28	0·137	31	0·184
20	0·223	28	0·164	32	0·171	30	0·124	45	0·110	47	0·139
42	0·179	45	0·158	60	0·117	60	0·080	59	0·096	60	0·096
79	0·133	80	0·112	110	0·091	90	0·094	75	0·084	75	0·093
109	0·107	110	0·085	165	0·090	120	0·092	93	0·087	91	0·100

α-Glucose.

Rabbit No.:	3
Exp. No.:	7
α-Glucose:	1·5 g.

Time mins.	Blood-sugar
Before	0·101
2	0·238
17	0·191
34	0·158
62	0·105
102	0·100
202	0·096

β-Glucose.

Rabbit No.:	6	7	3
Exp. No.:	23	24	16
β-Glucose:	1 g.	1·2 g.	1·5 g.

Time mins.	Blood-sugar	Time mins.	Blood-sugar	Time mins.	Blood-sugar
Before	0·088	Before	0·084	Before	0·091
8	0·221	6	0·186	6	0·226
30	0·132	35	0·100	21	0·181
45	0·093	55	0·100	36	0·156
60	0·107	75	0·098	61	0·110
75	0·099	113	0·096	91	0·076
120	0·095	—	—	181	0·085

The selected sugar was injected intravenously into the marginal vein of the ear in about 15 % solution in sterile distilled water. When α- or β-glucose was being used, care was taken that the injection was completed within 3 minutes of adding the glucose to the water, to minimise mutarotation. In most cases the injection was completed within 2 minutes. In the experiments with $\alpha\beta$-glucose, the sugar was dissolved in water at about 50° and allowed to stand about half an hour to ensure complete mutarotation. In most experiments a dose of sugar equal to 0·5 g. per kg. body weight was used. This gave a convenient rise in the blood-sugar. In some of the earlier experiments a dose of 1·5 g. was used, irrespective of body weight.

Any experiment in which any of the solution was injected outside the vein was discarded, and the results of such are not described above.

The blood-sugar estimations were made by the Hagedorn-Jensen method [1923], and are the means of closely-agreeing duplicate determinations.

The results are tabulated on p. 775.

The rabbits used in the above experiments weighed as follows: (1) 1700 g., (2) 1700 g., (3) 2500 g., (4) 2300 g., (6) 2000 g., (7) 2400 g.

Summary.

No definite difference was found between the behaviour of α-, β- and $\alpha\beta$-glucose when injected intravenously into rabbits.

The author's thanks are due to Sir F. G. Hopkins, at whose suggestion the work was undertaken, for his interest during its progress.

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XCVIII. THE ULTRA-VIOLET ABSORPTION SPECTRUM OF CHLOROPHYLL IN ALCOHOLIC SOLUTION.

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(*Received May 3rd, 1928.*)

Attention was drawn to the ultra-violet absorption spectrum of chlorophyll as a corollary to the spectrographic examination of olive oil [Lewkowitsch, 1927]. The latter gave distinct indications of selective absorption with a maximum at a wave-length 2670 Å.U., and the question arose whether this band was caused by chlorophyll, which in the visible range displays its own characteristic bands strongly marked in the absorption spectrum of olive (and some other vegetable) oils [Marcille, 1910].

The ultra-violet absorption spectrum of chlorophyll is unrecorded in the list of absorption spectra of organic compounds issued by the British Association in 1916, and the only account in the literature is a paper by Dhéré and Rogowski [1912]. These workers examined α- and β-"natural chlorophyll" from *Taxus baccata* and crystalline chlorophyll (from *Galeopsis tetrahit* by the combined methods of Monteverde and Willstätter) in solution in anhydrous ether; they obtained evidence of absorption maxima at λ 4300 and λ 3000–3100 Å.U. The method employed was to photograph the spectra transmitted through increasing thicknesses of solution.

Vlès [1919] calculated the anticipated spectrum in the ultra-violet of chlorophyll from an expression of the type $X = X_0 + An + Bn^2 + Cn^3$ from which he deduced the absorption band at 4320 Å.U.; his formula, however, did not provide for the band found by Dhéré at 3000 Å.U.

For the purposes of the present investigation a solution of chlorophyll extracted in the cold from dry powdered nettle leaves by alcohol was used. The solvent was purified for spectrographic purposes from absolute alcohol by distillation over iodine followed by distillation from excess of zinc dust. The solvent so prepared had practically no absorption down to a wave-length of about 2500 Å.U.; below this point the general absorption of the alcohol hindered examination of the chlorophyll.

The concentrated chlorophyll solution, stored in the dark, was used as a stock from which the more dilute solutions actually examined in the spectrophotometer were prepared immediately before use. Such a diluted solution was examined within one day, and the portion exposed to the light in the test-tube of the photometer was frequently renewed; these precautions were found necessary as the dilute solutions became rapidly decolorised, especially under the influence of the light of the spark.

The spectra were recorded by means of a Bellingham and Stanley spectrophotometer [Prideaux, 1926; Stanley, 1927] in conjunction with a quartz spectrograph. A condensed spark between iron electrodes was used as a source of ultra-violet light.

The spectrum obtained is shown in Fig. 1, in which "density," log. (incident light/transmitted light), is plotted against wave-length in Å.U. As the solutions examined were necessarily of arbitrary concentration, the figures for density are only relative values.

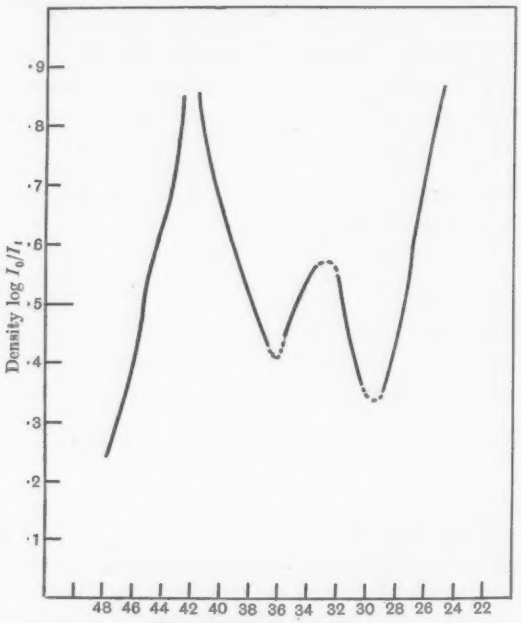

Fig. 1. Absorption spectrum of nettle chlorophyll in alcoholic solution.

Absorption maxima are shown at 4200 and 3250 Å.U., figures which agree reasonably with those of Dhéré; the slight difference in position of the maxima might be due to the effect of the different solvent used [Vogel, 1877; Van Gulik, 1915]. No trace could be found of any further minimum or descent of the curve which rose steeply below a wave-length of 2400 Å.U.

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XCIX. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VII.

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Report to the Medical Research Council.

(*Received May 1st, 1928.*)

In a previous contribution of this series [Zilva, 1927, 2] it was shown that the antiscorbutic activity in decitrated lemon juice is associated with a capacity for reducing phenolindophenol to its leuco-base. It was also found that when the juice had been heated in an autoclave at 120° under strictly anaerobic conditions for 1 hour, the antiscorbutic potency and the reducing capacity of the juice disappeared much more quickly on storage than it usually did in a similar juice which had not been previously autoclaved and which was stored under the same conditions. This phenomenon has since been subjected to further investigation in order to throw more light on the subject and some of the results are recorded in this communication.

Experimental.

The method of testing was the same as previously described. Three guinea-pigs weighing 250–300 g. were used for each dose, and doses equivalent to 1·5, 3 and 5 cc. of the original lemon juice were usually employed. The administration of the daily dose began after the animals had subsisted on the basal diet for 14 days. In the case of positive tests the guinea-pigs were chloroformed 60 days after the commencement of the experiment and the degree of scurvy, if present, assessed at the autopsy. With negative preparations, the animals, as is invariably the case on a basal diet of oats, bran and autoclaved milk, succumbed to scurvy within a month.

The instability of the antiscorbutic factor in the absence of the reducing agency.

When phenolindophenol is added to decitrated lemon juice until it is no longer reduced, the solution becomes alkaline owing to the hydrolysis of the indicator. The quick deterioration of the antiscorbutic activity in such solution on storage could, therefore, be ascribed either to accelerated oxidation in alkaline medium [Zilva, 1923] or to the removal of the protective action of the reducing principle. In order to test the second hypothesis it

was consequently necessary to adjust the reaction to neutrality very soon after the addition of the phenolindophenol. This is technically complicated by the fact that the electrometric measurement of the p_H in the adjustment of the reaction is tedious and long, especially as the presence of CO_2 and of carbonate in the medium requires the passing of hydrogen through the electrode for a considerable time before equilibrium is established. On the other hand, the use of p_H indicators in the adjustment of the reaction is complicated by the presence of phenolindophenol in the solution. This difficulty was, however, overcome in the following way. Ordinary decitrated lemon juice was adjusted to p_H 7. Solid phenolindophenol was then added cautiously until the indicator was no longer decolorised, *avoiding the addition of excess*. The p_H of the solution at this stage was approximately 8·4. A citric acid solution was now introduced without delay, drop by drop, until the p_H fell to 7, utilising simultaneously the comparator and capillator methods with phenol red as indicator. With practice, a fair degree of accuracy could thus be obtained. One portion of decitrated lemon juice treated in this way was fed *at once* to the guinea-pigs whilst another portion was stored in the *neutral condition* in the cold room for 24 hours before it was administered to the animals. All the 9 animals which received the stored preparation died of scurvy within a month. The control animals were chloroformed after 43 days, some signs of scurvy being established in the guinea-pigs on the lower doses. Excluding the detrimental action of the indicator on the vitamin *per se* this comparatively rapid inactivation of the decitrated lemon juice on storage can only be ascribed to the disappearance of the reducing activity, since untreated decitrated lemon juice stored under the same conditions at about p_H 7 for 24 hours loses comparatively little of its activity. The control experiment showed that the juice was active before storage. The animals in this set were chloroformed after 43 days as it has already been shown [Zilva, 1927, 2] that this treatment with phenolindophenol does not destroy the potency to any significant extent if tested immediately. The condition of the animals at the time of chloroforming showed that this was also the case in this instance. The post-mortem examination lent further confirmation of the activity of the preparation.

The stability of the antiscorbutic potency of comparatively pure fractions.

If the assumption is justified that in the chemical fractionation of the antiscorbutic factor substances are removed which contribute to the stability of the active principle, pure fractions might be expected to be less stable than the original juice. This was actually found to be the case. An active fraction was prepared from decitrated lemon juice by removing inactive material by clearing the juice with neutral lead acetate at p_H 5·4 and then precipitating the active principle by raising the p_H of the filtrate to 7 [Zilva, 1927, 1][1].

[1] Such preparations are usually active in doses equivalent to 1·5 cc. of the original juice in so far that the guinea-pigs survive on them the test period of 60 days. Occasionally such a degree of activity is not attained but the cause of this variation has not yet been ascertained.

Doses of this fraction equivalent to 5 cc. of the original juice were tested immediately after preparation, after 24 hours' and after 72 hours' storage in the cold room. Four guinea-pigs were used in each set. The animals to which the dose was administered immediately after preparation were chloroformed after 61 days, signs of mild scurvy being established at the post-mortem. Both sets of animals on the stored preparations succumbed to scurvy within a month, *i.e.* the stored doses showed no activity at all. The reducing capacity for phenolindophenol of the active fraction was about one-third of that of the original decitrated lemon juice from which it was prepared. In another experiment a fraction prepared as above was fed immediately after preparation and after 7 days' storage in doses equivalent to 1·5, 3 and 5 cc. of the original juice. In the former case the guinea-pigs survived for 60 days even on the lowest dose, whilst in the latter all the 9 animals, including those receiving the highest dose, succumbed to scurvy within a month. As ordinary decitrated lemon juice of similar antiscorbutic activity does not deteriorate so quickly one may reasonably infer that some substance or substances which have a protective action on the vitamin have been removed in the process of fractionation.

The dialysing properties of the reducing agency.

In view of the fact that, when decitrated lemon juice is dialysed for 3 days in a collodion thimble previously soaked in 92 % alcohol, it loses its antiscorbutic activity, whilst when a thimble which has been soaked in 88 % alcohol is used under the same circumstances comparatively little loss in activity takes place [Zilva and Miura, 1921; Connell and Zilva, 1924], it was of interest to study the behaviour of the reducing agency under these differential conditions of dialysis. Ordinary decitrated lemon juice was, therefore, dialysed as described before in "92 %" and in "83 %" collodion thimbles in the presence of succinic acid for 3 days. The dialysed juice, as well as a control juice kept in a test-tube in the dialysing tank, were tested by daily administration to guinea-pigs for their antiscorbutic activity, whilst the doses were titrated daily with phenolindophenol before being administered to the test animals. An "83 %" collodion thimble was used in the experiment because thimbles with an 88 % alcohol index made from this particular sample of collodion yielded a juice of low activity after dialysis. The juice dialysed in the "83 %" thimble showed some activity in 1·5 cc. doses and full protection for 55 days in the higher doses, as did also the control non-dialysed juice. The decitrated lemon juice dialysed in the "92 %" thimble, as was to be expected, was found to be inactive, even in 5 cc. doses. The phenolindophenol titrations revealed a total loss of the reducing agency in solutions dialysed in the "92 %" thimbles, an approximate loss of more than a half in the solutions dialysed in the "83 %" thimbles, and a loss of less than a quarter in the non-dialysed control. These observations suggest the necessity of revising the interpretation of the results in connection with the dialysing properties

of the antiscorbutic factor. It was previously assumed by the writer and his collaborators that because active solutions when dialysed through membranes with an alcohol index of 92–95 % became inactive, the vitamin had diffused out. Since, as we have seen from the above experiments, the reducing agency diffuses under these conditions, one cannot dismiss the possibility that the active molecule may be larger than was hitherto surmised and that by the diffusion of the reducing agency it becomes inactivated without actually diffusing out of the thimble. The matter requires further investigation.

The effect of acidity on the stability of the antiscorbutic factor in anaerobically autoclaved decitrated lemon juice.

In the previous communication it was shown that when decitrated lemon juice was autoclaved under strictly anaerobic conditions and stored, the antiscorbutic activity deteriorated quickly. The reaction of such solutions during storage was neutral. It was of interest to ascertain whether this deterioration on storage in the treated juice would also take place in an acid medium. The juice was, therefore, autoclaved as previously described, acidified to p_H 3 by the addition of citric acid and stored in the cold room for 7 days, after which time it was tested on the guinea-pigs. Seven out of the nine animals survived the test period of 62 days, showing little scurvy at the autopsy. Of the other two guinea-pigs, one, receiving a 1·5 cc. dose, died after 44 days, and the other, receiving a 3 cc. dose, died after 59 days from an intercurrent disease[1]. The control, non-acidified juice was found, as usual, to be inactive in 3 cc. doses. Experiments with autoclaved preparations stored at p_H 6 and at p_H 6·7 showed that the deterioration on storage was *definitely* retarded even at these hydrogen ion concentrations.

The effect of acidity during the process of autoclaving on the stability of the antiscorbutic factor and of the reducing agency in lemon juice.

Although an acid reaction retards the deterioration of the antiscorbutic activity of autoclaved decitrated lemon juice, it does not prevent, during autoclaving, the change which conduces to the accelerated deterioration of the active juice on storage. This was demonstrated by two experiments. In the first experiment decitrated lemon juice was brought up to p_H 6 by the addition of citric acid and autoclaved as before for 1 hour. After cooling it was adjusted to p_H 7 and kept at this hydrogen ion concentration for 7 days in the cold room before being tested. This stored preparation was found to be inactive even in doses of 5 cc. and had lost almost entirely its capacity for reducing phenolindophenol. In the second experiment freshly expressed lemon juice was autoclaved under strictly anaerobic conditions in the usual way. It was then decitrated, adjusted to p_H 7 and stored in this neutral condition in the cold room for 7 days before feeding. As a control, the autoclaved lemon

[1] These stored juices were found to reduce phenolindophenol but their reducing capacity was greatly diminished.

juice was tested *immediately* after decitration. Decitrated lemon juice autoclaved anaerobically at p_H 7, and administered at once and after 7 days' storage at p_H 7, was also tested at the same time. Although the guinea-pigs dosed on the autoclaved lemon juice immediately after decitration survived for 60 days even on the lowest dose of 1·5 cc., little activity was revealed by this juice when stored for 7 days at p_H 7. There was a slight delay in the death of the guinea-pigs receiving the higher doses, but all the 9 animals in the set died of scurvy within 30–40 days. As usual, decitrated juice autoclaved at p_H 7 and fed at once was found to be active, whilst after 7 days' storage the same juice failed entirely to protect in doses of 3 cc. The autoclaved lemon juice, immediately after decitration, reduced phenolindophenol almost to its full extent. On storage, this reducing capacity deteriorated to a very great extent, *i.e.* some of the stored specimens showed no reduction at all, in others, the titre was reduced to about a fifth or sixth of its original value.

The effect of heating to 143° *on the antiscorbutic factor in decitrated lemon juice.*

In this experiment decitrated lemon juice at p_H 7 was alternately evacuated and washed out with oxygen-free nitrogen three times and eventually autoclaved for 1 hour at 40 lbs. pressure (143°) in the evacuated ampoule. After cooling, the heated juice was tested at once. Of the guinea-pigs on the 1·5 cc. doses, one died after 44 days, another after 47 days and the third was chloroformed after 61 days. All the animals on this dose showed signs of scurvy at the post-mortem examination. Two guinea-pigs on the 3 cc. dose were chloroformed at the end of the test period of 61 days and were found to be free from scurvy. The third animal died of an intercurrent disease after 60 days, showing mild signs of scurvy. A similar result was obtained with the highest dose of 5 cc. Two of the animals were *fully* protected from scurvy for 61 days after which time they were chloroformed, the third animal died of an intercurrent disease after 60 days showing some signs of scurvy at the autopsy. It is, therefore, seen that, in spite of the very drastic treatment, the loss in the antiscorbutic potency of the juice was not great—a loss most probably due not to the thermal degradation of the vitamin but to the presence of very slight traces of oxygen or to other slight imperfections in the technique.

Conclusions.

The experiments dealt with in this communication are recorded with the object of lending further support to the suggestion made in the earlier paper previously quoted that the stability of the antiscorbutic factor in lemon juice is conditioned by the presence of a reducing principle and of a factor, the functioning of which is destroyed by heat. It was then suggested that the reducing property of the solution acted as a "reduction buffer" for the antiscorbutic vitamin. The results obtained in this investigation seem to justify this hypothesis. It is seen that after the reducing properties have been

destroyed the antiscorbutic activity does indeed disappear very much more rapidly. The dependence of the reducing agency, and consequently of the antiscorbutic factor, on the heat-labile factor is confirmed. Although acidity does not prevent the destruction of this last factor by heat, it exercises definite protection of the reducing and of the antiscorbutic principles after anaerobic heating. In fact, the change effected by autoclaving is such that the heated active solution becomes almost as unstable in the neutral zone as an unheated active solution is in the alkaline region—a change which is most probably due to an increased susceptibility to oxidation. We are thus faced with a vague picture of a complex mechanism in which the following points stand out in relief. The antiscorbutic factor, a principle which can withstand such drastic treatment as heating at 143° in the absence of air, becomes very labile in the presence of air, on changing the protective conditions of its natural medium. Experiments on purified fractions reveal that the process of chemical purification is one of the means of removing this protection.

As a mode of expression the writer has been compelled to employ such terms as "reducing agency" and "thermo-labile factor." This needs some qualification. The experimental evidence so far produced does not warrant the assumption of the existence of such substances in a preformed state in the cell and consequently the possibility of the presence of a large active molecule which, on destruction of the tissues of the plant, is degraded or modified and thus rendered less stable, must be borne in mind. This can only be decided by further work.

Summary.

When phenolindophenol is added to decitrated lemon juice until the indicator is no longer reduced and the solution is adjusted immediately to p_H 7, the antiscorbutic activity disappears within 24 hours.

Purified antiscorbutic fractions from lemon juice lose their activity much more rapidly than does decitrated lemon juice of similar activity.

Decitrated lemon juice dialysed in collodion thimbles of a permeability which leaves the solution inactive after 3 days, loses the capacity for reducing phenolindophenol. This reducing capacity is retained to a great extent by the juice when dialysed in thimbles of a permeability which yields an active juice at the end of the dialysis.

Acidity retards the deterioration, on storage, of the antiscorbutic activity in anaerobically autoclaved decitrated lemon juice. On storage at p_H 3 the deteriorating effect of autoclaving is hardly perceptible.

Lemon juice autoclaved anaerobically, even in a very acid medium, deteriorates much more rapidly at p_H 7 on storage than similar solutions which have not been autoclaved.

Comparatively little loss is registered in decitrated lemon juice which has been autoclaved at 40 lbs. pressure (143°) for 1 hour under *strictly* anaerobic conditions.

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C. FERMENTATION BY DRIED YEAST PREPARATIONS. II.

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It has been shown [Harden, 1925] that the addition of sodium acetate or bicarbonate accelerates the onset of fermentation by zymin in a large volume of sugar solution. Further investigation shows that this acceleration is produced by the presence of other salts, both organic and inorganic, the induction time varying with the concentration and with the specific nature of the salt.

To avoid irregularities previously noticed, the fermentations were always carried out in the following manner: 0·2 g. zymin was wetted for 1 minute with 1 cc. water; to this were added (1) the salt solution, (2) 0·5 cc. 20 % fructose, and (3) water to make a total volume of 2·5 cc. The mixture was saturated with CO_2 and incubated at 29·5°. A period of 5 minutes was allowed for the attainment of an even temperature, after which readings were taken at 5 minute intervals. In each series, a control flask containing zymin, sugar and water was incubated to give the normal induction time for this volume of sugar solution; this varied from 40 to 60 minutes according to the sample of zymin. For the purpose of comparison of the effects of various salts, the induction period has been fixed arbitrarily as the interval of time between the mixing and the attainment of a rate of fermentation of 0·2 cc. in 5 minutes.

The initial p_H of the mixtures after saturation with CO_2 was determined by the capillator method in duplicate flasks; the final p_H was also determined in the earlier experiments. No difference in the initial p_H on the addition of the salts could be detected, while that between the initial and final p_H was never more than 0·2, the initial p_H being 5·6.

The salts tested and the length of the induction period at the optimum concentrations found are given in Table I. With the exception of sodium arsenate, all the salts tested reduce the induction period materially, the optimum concentration, at least with the inorganic salts, appearing to be dependent on the nature of the anion. Thus the optimum varies from 0·1 *M* in the case of chlorides to 0·02 *M* for sulphates and phosphates and 0·002 *M* for sodium citrate. The last is peculiar in showing a sharply marked optimum concentration, for the other organic salts employed were effective within wide limits of concentration, and with less distinction between uni- and bi-valent anions.

Table I.

Salt	Optimum concentration (molar)	Minimum induction time (min.)
—	—	60
KCl	0·05–0·1	20
NaCl	0·1	25
NH_4Cl	0·05–0·1	20
K_2SO_4	0·01–0·05	20
Na_2SO_4	0·01–0·02	20
$MgSO_4$	0·01	20
Na_2HPO_4	0·02–0·04	20
K formate	0·2	25
K acetate	0·2	15
K propionate	0·05–0·4	10
K lactate	0·2	15
K succinate	0·05–0·1	20
K tartrate	0·05–0·1	20
Na citrate	0·002	20
Na glycerophosphate ...	0·04	20
Na hexosediphosphate ...	0·011 (minimum)	10
Na trehalosemonophosphate	0·016 (minimum)	15

Of the organic salts, potassium formate, tartrate, succinate, lactate and citrate have approximately the same effect as the inorganic salts; that is to say, the induction time is reduced from 60 to 20 minutes in their presence. Potassium propionate and acetate rank with the sodium salts of hexosediphosphate and trehalosemonophosphate in producing an immediate fermentation. The effective concentration of K propionate covers a wide range, 0·05 to 0·4 *M*, but one whose limits have been definitely ascertained. In the case of the hexosephosphoric esters, a minimum concentration of 0·01 *M* is necessary for the immediate onset of fermentation, but it has not yet been possible to determine whether a high concentration will eventually increase the induction time as is the case with other salts.

The position of sodium arsenate (Table II) with respect to this effect of salts is unique, in that it acts in a direction contrary to that of all the other salts tested. In concentrations as low as 0·00005 and 0·0002 *M*, the induction time is normal or slightly increased; as the concentration is raised, the induction time lengthens. This effect is all the more striking as, when fermentation does begin, the rate of fermentation is greatly increased above the normal, as is usual in the presence of arsenate. The length of the induction time has no deleterious effect upon the subsequent fermentation. This is again in contrast with the action of high concentrations of inorganic salts by which the induction time is prolonged and the subsequent rate of fermentation is diminished [Harden and Henley, 1921]. Furthermore, in the presence of arsenate, the beneficial effect of added salts, both organic and inorganic, is partially counteracted. Thus, in the presence of 0·1 *M* sodium chloride and 0·002 *M* arsenate, the induction time is 50 minutes, whereas that with sodium chloride alone is 20 minutes and with arsenate alone, 75 minutes. These results are shown in Table II.

Table II.

Induction time (min.) for 0·2 g. zymin in 2·5 cc. 4 % sugar solution in presence of arsenate ions and added salts.

Concentration of Na_2HAsO_4 (molar)	H_2O	0·1 *M* KCl	0·2 *M* Na acetate	0·002 *M* Na hexose-diphosphate
0	60	10	15	20
0·0002	65	20	—	—
0·002	75	45	45	55
0·01	120	—	—	—

The action of sodium arsenate in prolonging the induction time without any toxic effect on the ensuing fermentation appears to be specific; it is difficult, however, to link this with the only known effect of arsenate ions in the fermentation process, viz. an increase in the rate of decomposition of hexosediphosphate, more especially as the beneficial action of this salt is counteracted equally with that of the inorganic salts by the presence of arsenate in the mixture. It is possible, however, that the action of hexose-diphosphate and trehalosemonophosphate is in no way specific but falls in with the general effect of salts.

The phenomenon of induction under discussion presents some analogy to the induction which occurs in maceration extract and which has been studied by Meyerhof [1918]. In the latter case, however, the period of induction is completely abolished by the presence of hexosediphosphate in as small a concentration as 0·0002 *M*. It is moreover greatly diminished by the substitution of sucrose for glucose and fructose, which is not the case with the delayed fermentation of zymin. Meyerhof also found that the induction was removed by grinding the dried yeast with glass powder before preparing the maceration extract; grinding the zymin with glass powder, however, produced no effect on the induction period in the present experiments (see Table III).

Table III.

Induction time (min.) for 0·2 g. zymin in 2·5 cc. 4 % sugar solution.

Variation in conditions	Sugar		
	Fructose	Glucose	Sucrose
0·2 g. zymin unground	60	55	60
0·2 g. zymin + 0·1 g. glass powder	60	—	—
0·2 g. zymin ground with 0·1 g. glass powder	60	—	—
0·2 g. zymin + 0·2 cc. 1 % aldehyde... ...	50	—	—

The "induction" period in the case of zymin placed in a relatively large volume of sugar solution also appears to correspond to some extent with the period which elapses before the attainment of the maximum rate (Meyerhof's "Gäranstieg") in the fermentation of sugars in presence of phosphate by yeast preparations. Unlike this, however, it is very little affected by the addition of aldehyde (see Table III) so that it is probably not due to the same cause.

The results so far obtained render it probable that the induction period under discussion is due to the slow accumulation in the solution of one of the factors necessary for rapid fermentation. Whether this is accomplished by direct extraction from the zymin, a process which is aided by the presence of salts, or whether the process is a more complicated one, involving both the extraction and some subsequent chemical change before fermentation can proceed rapidly, remains to be ascertained.

SUMMARY.

1. It has been found that by adding various inorganic and organic salts to zymin placed in a large volume of sugar solution, the period of induction normally occurring before the onset of rapid fermentation is appreciably reduced.

2. Sodium arsenate is an exception to this general salt effect in that it prolongs the induction period without toxic effect on the fermentation.

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CI. THE DUAL NATURE OF WATER-SOLUBLE VITAMIN B. II.

THE EFFECT UPON YOUNG RATS OF VITAMIN B_2 DEFICIENCY AND A METHOD FOR THE BIOLOGICAL ASSAY OF VITAMIN B_2.

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In a previous communication [Chick and Roscoe, 1927], experiments were described which confirmed the conclusion of Goldberger and his colleagues [1925, 1926] that the water-soluble B vitamin as defined by the discoverers, McCollum and Davis [1915, 1, 2], had two components[1].

(1) Vitamin B_1 or the antineuritic, less heat-stable vitamin discovered by Eijkman in 1897, deficiency of which in diet leads to polyneuritis and death in birds, and to death, with or without paralysis, in rats and other mammals.

(2) Vitamin B_2, a more heat-stable vitamin, often accompanying vitamin B_1 in natural foodstuffs, in the absence of which the animal fails to grow and, in the case of rats, suffers from dermatitis.

Deficiency of vitamin B_1 is usually held to be the cause of beriberi in man, and, according to Goldberger and his colleagues, deficiency of vitamin B_2 is the cause of human pellagra.

When young rats are placed upon an experimental diet complete in all other respects, they show no growth if *either* of these two vitamins is lacking. In absence of vitamin B_1, death usually occurs in 3–4 weeks; in absence of B_2, the rats survive much longer. There is, therefore, no justification for calling vitamin B_2 a "growth factor" in contradistinction to vitamin B_1, as has been done by some writers.

That the water-soluble B vitamin and the antineuritic vitamin were not identical, as was supposed by McCollum and Kennedy [1916] and others, has long been suspected and a résumé of the literature on the subject was given in our previous paper [Chick and Roscoe, 1927]. Since the work of Goldberger and his colleagues was published, however, many papers besides our own have appeared which bear upon this point. In some, the separate action of vitamins B_1 and B_2 is clearly demonstrated, in others, it can be inferred.

[1] The nomenclature here used is in accordance with that provisionally adopted by the Biochemical Society.

Smith and Hendrick [1926] found that a synthetic diet devoid of water-soluble B vitamins was not rendered adequate to maintain growth in rats by adding Seidell's antineuritic concentrate (picrate) prepared from yeast, but required to be supplemented by an additional dietary factor. This second factor was also contained in yeast, and was able to withstand heating in the autoclave at 15 lbs. pressure for 6 hours.

Hauge and Carrick [1926], from observations on the growth and development of baby chicks, concluded that yeast contained a water-soluble vitamin necessary for growth which was separate from the antineuritic factor.

Salmon [1927] found that the seeds of the velvet bean were more potent than the leaves in preventing polyneuritis in pigeons fed on polished rice, but that the reverse was true for maintenance of growth in young rats when one or other of these two products was used to supplement experimental diets devoid of water-soluble B vitamins. Salmon succeeded in making a partial separation of the two water-soluble vitamins from the leaves of the velvet bean by adsorbing the antineuritic vitamin on fuller's earth, but in order to maintain growth in rats this antineuritic solid required to be supplemented with the filtrate yielded after treatment with the earth. In a later paper Salmon, Guerrant and Hays [1928] reported a more complete separation of these two principles to which they gave the names vitamin P–P, as suggested by Goldberger (= B_2) and B–P (= B_1). They found (1) that vitamin B_1 was adsorbed without appreciable admixture of vitamin B_2 if the extract made from the leaves were treated with a very small quantity of fuller's earth (10 g. per kg. air-dried leaves), and (2) that vitamin B_2 was precipitated with very small admixture of vitamin B_1, if alcohol to 82·7 % (by weight) were added to the leaf extract, after the latter had been previously treated several times with fuller's earth to remove vitamin B_1.

Williams and Waterman [1927], also using fuller's earth, removed a substance from an aqueous extract of yeast which was antineuritic but unable to maintain growth of rats on diets devoid of water-soluble B unless supplemented, for example, by yeast which had been heated for 6 hours at 125°.

Sherman and Axtmayer [1927] found that a supplementary relationship existed between whole wheat and autoclaved yeast and between whole wheat and dried skimmed milk when these were used to supply "vitamin B" to an otherwise complete diet. From these results the authors deduce the existence of two separate dietary factors in "vitamin B," both necessary for growth, for which they propose the names vitamin F (antineuritic) and vitamin G (heat stable).

In a series of recent papers Palmer and Kennedy [1927, 1, 2; 1928], have described the difficulties encountered in designing a "synthetic" diet, prepared from highly purified foodstuffs, which should be entirely satisfactory for growth and reproduction of rats. "Vitamin B" was supplied by an alcoholic extract of wheat embryo and highly purified caseinogen was the source of protein. The nutritive value was much improved when the diet was supple-

mented by autoclaved yeast or when commercial caseinogen was substituted for the highly purified product (see below, p. 793). It seems probable, though not proved, that vitamin B_2 is the missing constituent in their apparently complete "ration 5." This conclusion agrees with the results of recent investigations by Evans and Burr [1928] on the difference in growth of rats maintained on rations containing, respectively, purified caseinogen and sugar and the commercial products. They conclude that vitamin B_2 is present in the latter but not in the former. They also find that "Tikitiki," the dilute alcoholic extract of white rice polishings distributed to the natives by the Philippine Bureau of Science, possesses the antineuritic vitamin while "almost entirely lacking the growth-promoting vitamin B."

The technique which enabled us to demonstrate the separate effect on nutrition of these two vitamins was the use of the concentrated antineuritic vitamin prepared from yeast by the method of Peters [Peters, 1924; Kinnersley and Peters, 1925]. This product contains vitamin B_1 without any admixture of vitamin B_2 [Chick and Roscoe, 1927]. As a source of vitamin B_2, free from B_1, we used whole yeast autoclaved for 5 hours at 120°. This degree of heating destroys all the antineuritic vitamin contained in yeast, but only about one-half of the vitamin B_2 originally present.

Since our earlier paper was written some improvements in animal technique have been devised which have enabled us to make a more accurate study of the effect of vitamin B_2 deficiency on young growing rats. Incidentally, a method has been evolved for evaluation of vitamin B_2 which appears to possess a reasonable degree of accuracy for a biological method.

Improvements in animal technique required for study of vitamin B_2.

In our earlier experiments [Chick and Roscoe, 1927] we found that young rats on diet L[1] (devoid of water-soluble vitamins) and receiving vitamin B_1, in the form of a small daily ration of Peters's antineuritic concentrate, failed to grow and within 7–10 weeks developed the dermatitis and skin lesions described by Goldberger and Lillie [1926]. In subsequent experiments, however, skin symptoms were absent, although the animals were maintained for long periods on the diet and no significant increase in weight occurred. Other workers, *e.g.* Macy, Outhouse, Long and Graham [1927], have had the same experience.

By analogy with observations made upon other vitamins, it seemed possible that the irregularity might be due to variations in the reserves of vitamin B_2

[1] Diet L, devoid of water-soluble B vitamins:

Purified caseinogen	100 g.
Rice starch	300 g.
Cotton seed oil	75 g.
Salt mixture (McCollum's No. 185)	25 g.
Water	500 cc.

Cod-liver oil given daily by hand 0·05–0·1 g., according to the size of the rat.

The diet is heated, before use, for 3 hours in steam at 100°, in order to prevent the possibility of "refection" in the experimental animals [see Roscoe, 1927].

held by the animals. Although the young rat is incapable of storing any considerable quantity of vitamin B_1, the long period of survival when deprived of vitamin B_2 suggested a capacity for its storage.

Attempts were, therefore, made to reduce the supply of water-soluble B vitamins during lactation by limiting the mother's diet to rice, polenta (maize endosperm), white bread and milk, marmite being omitted. In case of one litter thus bred, 2 out of 3 receiving the "$-B_2$" diet developed the typical skin appearances; of these, one (No. 212) died, but the other (No. 213) showed some degree of spontaneous recovery. In the case of another litter, two rats (Nos. 172 and 178) survived more than 20 weeks without developing any skin lesions. These results showed no improvement in consistency and it was necessary to look elsewhere for the causes of the irregularity.

We next considered the possibility that our basal diet might not be sufficiently free from vitamin B_2. We had hitherto used caseinogen prepared commercially from skim milk by precipitation with acid, washing with acidulated water, drying and prolonged heating at 120°. The caseinogen was not extracted with alcohol, so that, while the heating to which it was subjected might have destroyed vitamin B_1, vitamin B_2 might still be present, and our irregular results might be due to the use of batches of caseinogen containing different amounts of B_2.

We therefore purified caseinogen as follows. "Light white soluble" caseinogen was dissolved to form a 5 % solution, reprecipitated with acetic acid and washed by decantation with 0·05 % acetic acid, the supernatant fluid being changed twice a day for a fortnight. The caseinogen was then extracted with dilute acidified alcohol (50–70 % strength (by volume), containing about 0·03 % acetic acid) for 96 hours in a large Soxhlet apparatus, and afterwards washed with 93 % alcohol, dried in an electric oven at a low temperature, ground in a mill and finally roasted for 3 days at 120°.

The behaviour of young rats 40–50 g. in weight receiving the "$-B_2$" diet prepared with this highly purified caseinogen was compared with that of others from the same litter receiving a similar diet made with the caseinogen previously used. After 12 weeks the difference between the two sets of rats was striking. Two rats receiving the commercially purified caseinogen had poor coats and had shown no significant increase in weight (weight 52 g.) but they were in fair condition, they showed no skin symptoms, and their eyes remained normal (see rats 218 and 222, Fig. 1). The weight of 7 rats receiving the specially purified caseinogen ranged from 39 to 47 g., 5 showed typical skin symptoms, all had sunken eyes and inflamed eyelids and their general condition was miserable (see rats 217, 220 and 221, Fig. 1).

It is evident, therefore, that vitamin B_2 is present in precipitated caseinogen, and can be removed by thorough extraction with acid water and acid alcohol. This is a point of some importance and, in part at least, may account for the high nutritive value possessed by caseinogen as a protein. It probably explains the improvement observed by Palmer and Kennedy [1927, 2] in the nutri-

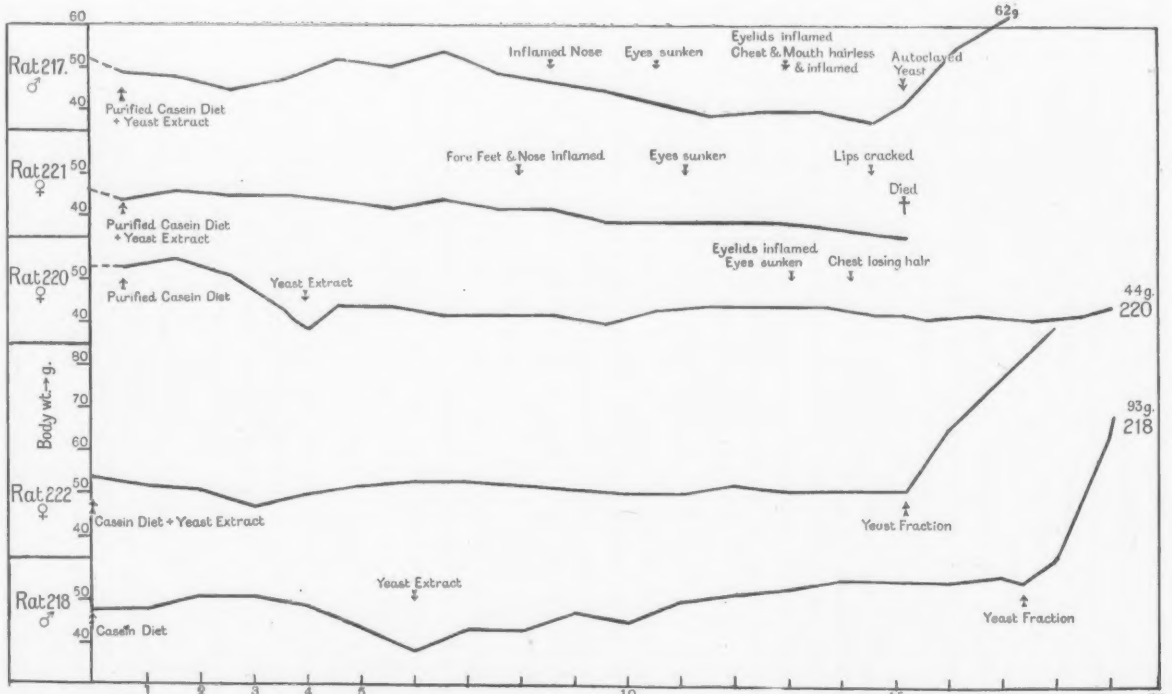

Fig. 1. Weight charts of rats on diets devoid of water-soluble B vitamins.
Yeast extract contains B_1. Yeast fraction contains B_2.

tional value of their "synthetic" ration for rats, when a commercial preparation of caseinogen was substituted for their highly purified product. The disappointing results obtained by Goldberger and Tanner [1925] with highly purified caseinogen, as compared with milk and milk products, when these were added to the diet of inmates in Institutions where pellagra was endemic, may possibly have a similar explanation.

Symptoms shown by young rats on a diet deprived of vitamin B_2.

Young rats can often be maintained for 3 months and longer upon a diet deprived of vitamin B_2. During this period they will show no significant increase or loss in weight (see Fig. 1). They do not, however, appear to be ill or distressed, they have a fair appetite and remain active and interested in their environment. The skin symptoms, which are symmetrically placed on the animal, gradually develop at any time after the 5th or 6th week, but do not usually become severe until several weeks later. The most constant symptoms in our experience are the following. (1) Dermatitis and loss of hair from the eyelids, which may become stuck together; if the eyelids are loosened by bathing with warm water, the eyes, though much sunken, appear to be healthy. (2) Front paws stained with blood, caused by rubbing the inflamed margins of the nostrils; wetting of the lower portion of the abdomen with blood stained urine. (3) Dermatitis and loss of fur on head, round the nose and mouth and on the abdomen. Inflammation of the skin at the tips of the ears and a curious and very characteristic oedematous dermatitis of the digits of the paws, which become bright red in colour, were constant early symptoms in our earlier experiments, but at present are encountered later and much less frequently.

The irregularity in the occurrence of the various skin symptoms and the occasional instances of animals deprived of vitamin B_2 remaining stunted in growth but exhibiting no special skin lesions, suggest that we may possibly be dealing with more than one kind of dietary deficiency. There has certainly

Fig. 1. Weight charts of 5 young rats from the same litter on basal diets devoid of water-soluble B vitamins. Vitamin B_1 given as Peters's concentrated antineuritic extract from yeast (daily dose $\equiv$0·6 g. dry yeast). Rats 217, 220, 221 and rats 218, 222, received diets prepared from the more and less highly purified caseinogen respectively.

Rat 217 (initial weight, 52 g.) received vitamin B_1 throughout, developed typical skin lesions after 9 weeks; at 15 weeks (weight 39 g.) received vitamin B_2 as 0·4 g. daily of yeast autoclaved for 5 hours at 120°.

Rat 221 (initial weight, 46 g.) treated similarly to rat 217, developed skin lesions after 8 weeks, remained untreated and died after 15 weeks of vitamin B_2 deficiency (weight 35 g.).

Rat 220 (initial weight 53 g.) at first deprived of both B vitamins, showed collapse (weight 38 g.) due to lack of vitamin B_1 at $4\frac{1}{2}$ weeks, was restored by administration of vitamin B_1, developed skin lesions after the 13th week (weight after 19 weeks, 44 g.).

Rat 222 (initial weight 54 g.) received vitamin B_1 throughout; at 15 weeks had developed no skin symptoms (weight 51 g.); administration of vitamin B_2 in the form of a yeast fraction, caused increase in weight of 39 g. in 3 weeks.

Rat 218 (initial weight 48 g.) at first deprived of both B vitamins, showed collapse (weight 38 g.) due to lack of vitamin B_1 at 6 weeks, restored by administration of vitamin B_1, showed no skin symptoms in 18 weeks (weight 54 g.); administration of vitamin B_2, in form of a yeast fraction, caused immediate increase in weight (46 g. in 2 weeks).

been greater constancy in the development of symptoms since we have taken steps to free the caseinogen in the basal diet from possible contamination with water- or alcohol-soluble vitamins, but, on the other hand, the typical dermatitis of the paws and ears, for example, which is now so infrequent, was previously often observed in rats upon diets prepared with less pure caseinogen and receiving a small, though insufficient, supply of vitamin B_2 in the form of autoclaved yeast [see Chick and Roscoe, 1927, Table II].

Our experience also differs in some respects from that of Findlay [1928] who records "moderate growth...for from 4 to 6 weeks" in rats deprived of vitamin B_2, followed by "slight but gradual loss of weight" with development of skin lesions. We have not observed the change in temper of the animals, those previously tame and docile becoming irritable and liable to bite, described by him, nor have we observed any great loss in appetite. In our experience, the intake of food remains rather high (about 4–6 g. daily dry weight) seeing that the body weight of the animals remains steady at about 40–50 g.

Recovery on addition of vitamin B_2 to the diet.

If the condition of the rats after many weeks on the "$-B_2$" diet is not too bad, they respond promptly to administration of vitamin B_2 and increase in weight begins immediately, often within 24 hours. If the supply of vitamin B_2 is abundant the growth in weight may be far in excess of the normal for a time and we have frequently observed gains of 20–30 g. a week for a short period (see Table I and Curves of rat 218, Fig. 1). The sunken eyes are usually the next to show improvement and soon begin to protrude. Desquamation of the affected skin also takes place, after which the fur begins to grow and the animal gains a normal appearance within 2–4 weeks, according to the severity of the previous symptoms. It remains for some time undersized for its age.

Method for evaluation of vitamin B_2.

If the vitamin B_2 is supplied after only a few weeks of deprivation and before the animal's condition is too bad, the response in growth is at first roughly in proportion to the amount of vitamin B_2 administered. It is well, however, not to leave the animal too long on the deficient diet (see rat 251, Table I) and there is no need to await the development of specific symptoms.

Young rats are placed upon the basal diet, deprived of B vitamins, when about 4 weeks old and 40–50 g. in body weight. They are left together in a large cage for about 10 days. After this time, they are placed in separate cages, because of their tendency to pull out and eat the fur of their companions. The cages have floors of wide-meshed ($\frac{1}{3}$-inch) zinc gauze, raised about 2 inches above trays filled with peat moss litter. This precaution is necessary in order to hinder the consumption of the faeces, which is a common practice of rats on diets deprived of B vitamins, and tends to obviate the effect of the dietary deficiency [Steenbock, Sell and Nelson, 1923].

Table I. *Titration of vitamin* B_2 *by increase of body weight in young rats, which have ceased to grow on "* $-B_2$*" diet, i.e. diet* L, *devoid of* B *vitamins, to which vitamin* B_1 *is added as Kinnersley and Peters's concentrate from yeast.*

Material tested	Daily dose expressed as the equivalent of dry yeast g.	Rat No.	Body weight when dose was started g.	Weekly growth increments while receiving dose g.	Mean for rats receiving a given dose g.	Time previously maintained on "$-B_2$" diet Weeks
Fraction 5 I, extract of Yeast V in 0·01 % acetic acid evaporated to small bulk	1·2	142	116	36, 32	26	14
	1·2	140	101	24, 12		14
	0·9	243	45	26, 22	24	3
Fraction 5 II, prepared as 5 I, from Yeast V and VII	0·5	254	41	21	21	2
	0·5	255	41	22		2
	0·5	256	43	19		2
	0·25	254	71	14, 11	11	2 + 2 on above test + 2 deprived until weight was again constant
	0·25	255	65	14, 12		
	0·25	256	66	8, 11		
Fraction 5 III, prepared as 5 I, from Yeast VIII	1·0	251	45	24, 28	26	15 + 1 receiving the equivalent of 0·5 g. yeast with no response
	0·5	251	43	2	—	15
	0·5	268	52	20, 15·5	18	6
	0·25	272	53	10·5, 13·5	11	7
	0·25	279	38	16, 17		4
	0·25	280	39	11, 11		2
	0·25	273	56	11, 9, 5, 9·5		2 + 2 receiving the equivalent of 0·12 g. yeast
	0·12	273	46	7, 3	7	2
	0·12	290	37	9, 12, 3		2
Dried Yeast	0·4	—	—	—	23	See Chick and Roscoe [1927], Table III
	0·2	—	—	—	11	

The daily dose of Peters's antineuritic concentrate is omitted at first, for reasons of economy, but after about 2 weeks, it is given. A daily dose of 0·1 cc. (equivalent to 0·6 g. of the original yeast, dry weight) provides an excess of vitamin B_1. It is necessary to remove all traces of lead and mercury from the preparation, seeing that it is to be fed regularly over long periods. This is conveniently done after the precipitation with Hopkins's reagent (acid mercuric sulphate) and before adsorption on norite charcoal. If precipitation with baryta is inserted after treatment with lead acetate, as recommended recently by Kinnersley and Peters [1927], the separation of the metallic sulphides is much facilitated.

The weight of the rat sometimes showed a temporary increase of a few grams after this inclusion of vitamin B_1 in the diet. After 3–4 weeks from the beginning of the experimental feeding, however, the young rat is prepared for testing the content of vitamin B_2 in any material which may be then administered. The minimum dose which gives an average weekly increase of 10–12 g. provides a convenient standard for comparison. It is not necessary

for the material tested to be freed from vitamin B_1, of which an excess is already present in the rat's diet.

Table I shows the results of titrating the vitamin B_2 content of a yeast extract made with 0·01 % acetic acid, the first filtrate obtained in the Kinnersley and Peters process for preparing an antineuritic concentrate from yeast. The tests were made on extracts from three different samples of yeast and show concordant results as long as the animals used have not been longer than 6–7 weeks on the deficient diet (see rat 251). For example, rat 268, which had been 6 weeks on the diet devoid of vitamin B_2, responded to a daily dose of Fraction 5 III, equivalent to 0·5 g. dry yeast, by an average increase in weight of 18 g. a week, whereas rat 251, after 15 weeks on the deficient diet, made no significant response to the same dose. This animal, however, grew 26 g. a week when it received the equivalent of 1 g. dry yeast. From the results obtained with rat 273 when testing Fraction 5 III, it appears justifiable to use the same rat successively for assay of two different doses of vitamin B_2, if the smaller dose is given first. If a larger is given first, an interval of 2 weeks without any dose should intervene before the smaller one is tested (see results of tests made with rats 254, 255 and 256 on Fraction 5 II).

The results obtained with Fractions 5 I, 5 II and 5 III, compared with those obtained with dried yeast, show that little loss of vitamin B_2 has taken place during the extraction.

Summary.

1. Additional evidence is given proving the existence of the two separate vitamins B_1 and B_2 in the "water-soluble B" vitamin complex (for definitions see p. 790).

2. An improved animal technique is described for the study of vitamin B_2, the most important modification being the use of highly purified caseinogen as source of protein in the basal diet. The antineuritic concentrate prepared by the method of Kinnersley and Peters is used as source of vitamin B_1.

3. A description is given of the effects observed in growing rats when they are fed on diets deficient only in vitamin B_2.

4. A method is described for assay of vitamin B_2.

5. It is suggested that the high value of caseinogen as a protein may be partly due to contamination with vitamin B_2 and that the accepted nutritional value for this and other proteins may need revision.

Our thanks are due to Sir Charles Martin for his constant advice and criticism.

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CII. STUDIES ON LIPIN-PROTEIN COMPLEXES.

I. LECITHIN-CASEINOGEN COMPLEXES.

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It has long been known that lipins and proteins form loose "compounds" with each other, but up to the present no certainty seems to have been reached as to whether the constituents of these compounds are united merely by surface forces or whether their association is of a more intimate chemical nature. Galeotti and Giampalmo [1907] attempted to decide between these alternatives. They found on mixing lipins and proteins in various widely differing proportions that the composition of the resulting precipitates (as judged by the nitrogen content) although varying between only relatively narrow limits was not sufficiently nearly constant to enable them to deduce with certainty the formation of any definite chemical compound. In the same year, however, Mayer and Terroine [1907] studied the mutual interaction of lecithin and albumins and as a result stated definitely that the precipitates obtained on acidifying mixtures of these substances are colloidal complexes. Later Feinschmidt [1912] investigated the precipitation of mixtures of lecithin and various proportions of serum-proteins in solutions of graded acidity, and showed that the optimum hydrogen ion concentration at which the maximum precipitation of such mixtures occurs lies between the respective optimum values observed for the separate constituents alone. Meanwhile Hardy and Gardiner [1910] and Handovsky and Wagner [1911] pointed out the occurrence of lipin-protein complexes in blood-serum, or at least in the precipitates obtained therefrom by the addition of acids or salts, and following this Chick [1914] showed that the euglobulin precipitated by these reagents from serum is itself such a lipin-protein complex. Michaelis [1926] quotes a typical experiment of the kind described by Feinschmidt, and on repeating this we noticed that the precipitates produced in the relatively more acid solutions tended to float to the surface of the liquid, and so appeared to contain a larger proportion of the lighter lipin constituent than those produced in the less acid range, which were seen to settle more quickly to the bottom. As we had already been confronted with the question [Parsons and Parsons, 1927] as to the extent to which the lipins and proteins of serum are united into complexes, and as

to the conditions of precipitation of these complexes by salts, it seemed desirable to undertake a detailed investigation of the formation and properties of such complexes in general, and it is the object of the present paper to record the results of the first experiments made to this end.

In these experiments it was decided to use caseinogen as the protein constituent, on account of the ease with which it can be obtained pure (and, in particular, fat-free), while Merck's lecithin from eggs was chosen as the lipin component. In order to render possible the completion of the determinations on any one series of mixtures within a practicable length of time it was necessary to employ colorimetric methods of analysis. In order to obtain such a method for the estimation of the amount of lecithin present in a precipitated complex we stained this substance before use with a lipin-soluble but water-insoluble dye. When a precipitate was to be analysed it was first treated with a suitable volume of absolute alcohol in which the lecithin and the dye were at once dissolved. A measure of the amount of lecithin in this solution was then obtained by comparing the depth of its colour with that of an alcoholic solution of a known amount of the original stained lecithin preparation. Sudan III proved to be a suitable dye for this purpose. In order to obtain a uniformly stained lecithin preparation the lipin and a suitable small quantity of the dye were dissolved together in a little ether which was then allowed to evaporate. The resulting stained lecithin was then employed for the preparation of the emulsions used in the experiments described below. Of course it is admitted that the presence of the dye might to a certain extent influence the colloidal behaviour of the lecithin; but that such an influence is of appreciable magnitude in these experiments is hardly to be expected, on account of the smallness of the quantity of dye used, and this expectation is further justified by our observation that the point of maximum precipitation of the stained lecithin in the absence of protein corresponds with the hydrogen ion concentration at which the unstained lecithin itself is most readily precipitated from its emulsions. We intend to investigate further the question of the possible small effect of the dye by comparing the quantitative behaviour of deeply stained with lightly stained preparations and by the use of various staining materials. But meanwhile the experimental results recorded below must be understood to refer to lecithin containing the small quantity of Sudan III requisite for its estimation by the method outlined above.

For the colorimetric estimation of the caseinogen in the precipitates the method devised by Wu [1922] for the estimation of serum-proteins was found to be applicable. In this a phosphomolybdotungstate reagent is used which gives a blue colour with phenols generally, and so with the tyrosine of the protein molecule. The protein remaining after the complete alcohol extraction of the lipin from the original precipitate was dissolved in an appropriate volume of saturated sodium carbonate solution. After suitable dilution of this liquid the phenol reagent was then added, and when the resulting colour had

acquired a suitable and practically constant intensity it was compared with that obtained in a standard caseinogen solution treated in the same way simultaneously with the unknown. As a practical detail it may be mentioned that for a quantity of caseinogen of the order of 0·002 g. it was convenient to use 1 cc. of saturated sodium carbonate solution, to dilute then with 5 cc. of distilled water and to add 0·1 cc. of Wu's reagent.

In the first place it was decided to obtain information as to the variation of composition of the lecithin-caseinogen complexes precipitated from mixed colloidal solutions of these substances at hydrogen ion concentrations intermediate between their respective isoelectric points. The starting materials were a 0·4 % solution of caseinogen made up in 0·1 *N* sodium acetate according to the directions given by Michaelis [1926] and a 1 % emulsion in distilled water of Merck's lecithin stained with Sudan III. The necessary range of hydrogen ion concentration was obtained by the use of lactic acid-sodium lactate buffer solutions also made up according to Michaelis, and contained in 30 cc. portions in centrifuge tubes. To each of one series of tubes were added 3 cc. of a mixture of 1 volume of the lecithin emulsion with 2 volumes of the caseinogen solution. For comparison, each tube of a second similar series received 3 cc. of a mixture of 1 volume of lecithin emulsion with 2 volumes of 0·1 *N* sodium acetate solution while a third series was made up with 2 cc. of caseinogen solution + 1 cc. of distilled water in each tube. Thus the total volume of fluid and the concentration of sodium acetate were identical for all the tubes in the three series.

After standing for a period of 48 hours the precipitates of lecithin-caseinogen complex in tubes p_H 3·6 to 1·4 were aggregated by light centrifuging and the supernatant liquids were cautiously sucked off. Each precipitate was then extracted with two successive portions of 5 cc. of absolute alcohol, the alcoholic extracts were carefully decanted from the caseinogen residues and the depths of colour of the combined extracts were compared in tubes of equal diameters with those of a series of standards prepared by suitable dilution of an alcoholic solution containing in 10 cc. the same amount of stained lecithin as had been added to each of the buffer solutions. The caseinogen residues were dissolved each in 2 cc. of saturated sodium carbonate solution + 10 cc. of distilled water, 0·2 cc. of Wu's phenol reagent was added to each tube and after an hour's standing the resulting blue colours were compared in a similar way with those produced in a series of suitable dilutions of the original caseinogen solution simultaneously treated in the same fashion. The results of these determinations, together with those of the similar ones carried out on the pure lecithin and caseinogen precipitates obtained in those tubes that received these substances alone, are represented graphically in Figs. 1 and 2, of which the first shows the actual amounts of lecithin and caseinogen precipitated together at the various hydrogen ion concentrations and the second the increase in the percentage of lecithin in the precipitate with increasing acidity of the solution. It will be seen that the change in composition of the

precipitated complex is really very considerable over the range investigated, the percentage of lecithin increasing from 27 at p_H 4·2 to 100 at p_H 1·4. The increase, however, is not regular, for over the intermediate portion of the range—from p_H 3·6 to p_H 2·9—the percentage of lecithin remains practically constant at a value of about 55. By comparing the two figures it will be seen that this range of constant composition of precipitate corresponds with the range over which all the lecithin and caseinogen present are thrown out of solution. It is conceivable, therefore, that over this range the caseinogen was capable of combining with more lecithin than was actually present, and that

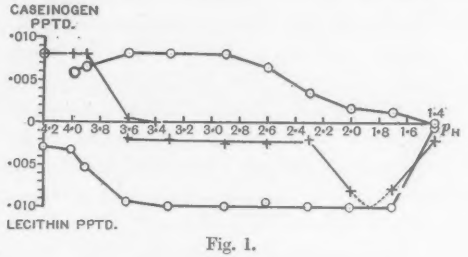

Fig. 1.

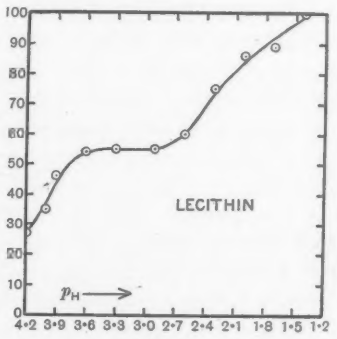

Fig. 2.

if we had started with a rather larger proportion of the lipin constituent this region of constant composition of precipitate, and with it the inflexion in the curve of Fig. 2, would have been absent.

It is probable that we are dealing here with a case of the mutual precipitation of two oppositely charged colloids, a phenomenon that has been familiar since the investigations of Biltz [1904]. In this case all the solutions which we used possessed hydrogen ion concentrations greater than that corresponding to the isoelectric point of the caseinogen so that this carried a positive charge, while the lecithin in the same solutions was on the alkaline side of its isoelectric point and so carried a negative charge. Presumably when the oppositely charged colloids are present together in solution the charge on the particles of one tends to be neutralised by the attraction of oppositely charged particles

of the other, and, when this process of neutralisation has resulted in the formation of complexes whose charge, although not necessarily zero, is too small to keep them in solution by mutual repulsion, precipitation occurs. In order to arrive at a quantitative explanation of the results of this experiment it is necessary to possess also some information as to the manner in which the charges on the two colloids which we have employed vary with the hydrogen ion concentration of the dispersion medium. In the case of the caseinogen it may be supposed that the charge on its particles is derived by a process of ionisation and that this ionisation is the more complete (or the activity of the caseinogen ions is the greater) the more remote the hydrogen ion concentration of the solution from the isoelectric point. But we should expect that once a certain hydrogen ion concentration has been reached the ionisation would be complete and that further increase of acidity would be without appreciable effect on the charge on the caseinogen particles. With regard to the lecithin there seems to be less evidence as to how the charge on its particles varies in relation to the reaction of the medium of dispersion. In this case the charge is possibly determined to a great extent by factors other than ionisation so that it might be expected to increase continuously with diminution of hydrogen ion concentration of the solution in a manner somewhat similar to that recently demonstrated by Michaelis and Dambo-viceanu [1924] in the case of mastic particles. If we assume that such relationships apply to the two colloids here under investigation it is possible to explain the proportions in which they mutually precipitate one another at various reactions. For in the less acid solutions the caseinogen particles will be but feebly positively charged while the lecithin will carry a relatively high negative charge. In this case, therefore, the adsorption of a comparatively small amount of the lipin will suffice to reduce the charge on the caseinogen particles to a point at which the complex will be precipitated. Thus the complex precipitated at these reactions will be rich in caseinogen and correspondingly poor in lecithin. On the other hand in the more acid solutions, the charge on the lecithin being less and that on the caseinogen being greater, relatively more lecithin will be required for neutralisation to the precipitating point and the complex obtained under these conditions will consequently contain a high proportion of lecithin. And this is indeed what is actually found to be the case. At first sight it would seem that on this theory the change of composition of the precipitated complex must be continuous over the range of hydrogen ion concentration between the isoelectric points of the two constituent colloids but since exact neutralisation of charge is probably not essential to mutual precipitation it is easy to account for the precipitates of practically constant composition obtained over a certain range of hydrogen ion concentration on the ground that not all of them were exactly isoelectric. In the more acid solutions the lecithin is too feebly charged to bring down more than a small quantity of the caseinogen; in fact in these solutions the lipin is precipitated when present alone and the protein appears

to exert no protective action on it. On the other hand in the least acid solutions the lecithin appears to be so strongly adsorbed by the caseinogen that a reversal of charge takes place and the resulting complex is therefore sufficiently negatively charged to remain in dispersion, for less of the protein is precipitated in the presence of the lipin than would be precipitated in its absence, a protective action being observed in this case.

We obtained further information regarding the mutual precipitation of these colloids by studying the composition of the complexes obtained when they were added in widely differing proportions to a buffer solution whose hydrogen ion concentration was so far intermediate between those corresponding to the isoelectric points of lecithin and caseinogen respectively that in it neither of these constituents was precipitated if present alone. For this purpose a lactic acid-sodium lactate mixture of p_H 2·7 (standardised electrometrically) was chosen. As before we used a 1 % emulsion of stained lecithin in water and a 0·4 % solution of caseinogen in 0·1 *N* sodium acetate solution. 1 cc. of a mixture of 1 volume of the lecithin emulsion + 2 volumes of the caseinogen solution was first prepared: this gave a mixture denoted by the symbol $\frac{L}{1}$ which has the same composition as that used for the experiment just described. A second mixture, $\frac{L}{2}$, was made by adding 1 cc. of a twofold dilution of the original lecithin emulsion to twice its volume of caseinogen solution, and by using further successive twofold dilutions of the emulsion other mixtures, $\frac{L}{4}$, $\frac{L}{8}$ and $\frac{L}{16}$, were prepared containing the same concentration of caseinogen throughout but diminishing quantities of lecithin equal respectively to $\frac{1}{4}$, $\frac{1}{8}$ and $\frac{1}{16}$ of the amount present in mixture $\frac{L}{1}$. A second series of mixtures, $\frac{C}{2}$, $\frac{C}{4}$, $\frac{C}{8}$, $\frac{C}{16}$, was also prepared using a constant amount of lecithin and twice its volume of successive twofold dilutions with 0·1 *N* sodium acetate solution of the original caseinogen dispersion, so that a total series was obtained in which the relative ratio of lecithin to caseinogen varied from $\frac{1}{16}$ to 16. Of each mixture in the middle of the series 1 cc. was added to 10 cc. of buffer solution, but of the more extreme mixtures, $\frac{L}{8}$, $\frac{L}{16}$, $\frac{C}{8}$ and $\frac{C}{16}$, 3 cc. were added to 30 cc. of buffer solution in order to obtain a larger amount of precipitate for analysis. It was noticed that the mixtures containing the greatest proportion of lecithin precipitated first. After a period of 48 hours the precipitates were aggregated by light centrifuging and analysed by the methods already described, care being taken to treat the standards identically with the unknowns and to obtain as accurate readings as possible by making the comparisons by means of a colorimeter.

From the results so obtained we calculated the amounts of caseinogen and lecithin precipitated in the various complexes per unit quantity (0·0033 g.) of total lecithin originally added to the mixture: these quantities are plotted in Fig. 3 in which positive ordinates represent g. of caseinogen, and negative

ordinates g. of lecithin, while the proportions in which these two constituents were added to the buffer solution are indicated by equally-spaced abscissae, *i.e.* by distances proportional to the logarithms of the actual numerical ratios concerned. The total amounts of the constituents either originally taken or appearing in the precipitated complexes are expressed by the correspondingly labelled curves.

Fig. 3 furnishes additional evidence in support of the view previously put forward that precipitation of the lecithin-caseinogen complex takes place only when the two components have combined in such proportions that mutual neutralisation of their opposite electric charges occurs to an extent sufficient to reduce the final charge on the particles of the total complex, if not neces-

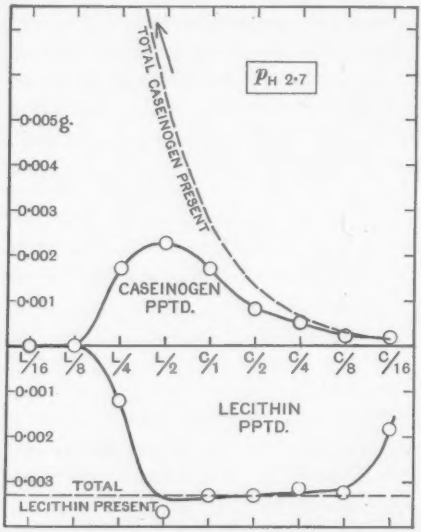

Fig. 3.

sarily to zero, at least to a point below a certain critical value. The first feature to be noted is that at the highest caseinogen concentrations (on the left of the figure) no precipitation at all of either component occurs. In this region we may suppose that the complex formed in solution contains so high a proportion of the positively charged caseinogen that the mutual electrostatic repulsion even of those complex particles that are relatively most feebly charged is powerful enough to maintain the condition of permanent dispersion. We are evidently dealing here with the same phenomenon encountered by Mayer and Terroine [1907] when they found that their lecithin-albumin complexes were soluble in excess of the protein constituent. As the amount of caseinogen present is diminished we come to a point at which precipitation of complex just begins. Here we must suppose that while most

of the particles of the colloidal complex are still too rich in caseinogen, and therefore too highly positively charged to be precipitated, a certain small fraction of them, containing a relatively smaller proportion of the protein than the others, carry less than the critical intensity of charge and so are precipitated. As we proceed further we encounter the, at first sight surprising, circumstance that as the total amount of caseinogen present is still further reduced the amount of the protein in the precipitate steadily increases, which evidently means that the reduction of the percentage of caseinogen in the complex formed over this range is more than compensated by the extra amount of precipitation permitted by the concomitant reduction of charge. This increased precipitation of caseinogen continues until a certain maximum amount is reached in a mixture which is the first from which the whole of the lecithin present appears in the precipitate. With this particular ratio of the constituents we therefore obtain the maximum total amount of precipitate. Beyond this point further reduction in the total amount of caseinogen present leads to a reduction in the amount of this component precipitated, until, in the smallest caseinogen concentrations we at last arrive at a point at which there is not a sufficient proportion of the protein in all of the complex particles to reduce the negative charge due to the lecithin to below the negative critical precipitation value, so that only the particles relatively poor in lecithin are precipitated and a portion of this substance now again remains in solution. Finally we approach the condition in which practically no caseinogen is present, and no lecithin is precipitated.

Somewhere between the point at which all the added lecithin just begins to appear in the precipitate combined with the maximum amount of caseinogen, and that at which, in the mixtures poor in caseinogen, the whole of the lecithin just ceases to be precipitated, there must occur the isoelectric complex in which the charge, although not necessarily zero for all particles has a minimum average value. But from the results of this experiment alone it is not possible to discover which this ratio is. We are not justified in assuming that the isoelectric ratio is that in which the constituents are combined when the total weight of the resulting complex precipitate is a maximum, *i.e.* in the precipitate in which the whole of the lecithin appears associated with the maximum amount of caseinogen. For, as we show in Fig. 5, this precipitate does not contain a maximum *percentage* of caseinogen; it is but one of a series of precipitates whose caseinogen percentages diminish in a regular continuous fashion, so that all we can say is that the particles of this particular complex are, on the whole, less powerfully positively (or more powerfully negatively) charged than the particles of the complex obtained from mixtures richer in caseinogen, and conversely more strongly positively (or less strongly negatively) charged than those given by succeeding mixtures poorer in caseinogen.

An experiment was also carried out in which lecithin-caseinogen mixtures in the same ratios as those used in the experiment just described in detail were added to a less acid (lactic) buffer solution of p_{H} 3·7 in which caseinogen

itself is precipitated when present alone. The results of this experiment in general confirm the deductions already drawn from a study of the behaviour of these constituents in the more acid solution. Attention is directed to the differences in detail produced by the circumstance that the caseinogen in the medium of p_H 3·7 is less powerfully positively, and the lecithin more powerfully negatively, charged than in the solution previously used. In the first place it was noted that at p_H 3·7 the mixtures containing the greatest proportion of caseinogen precipitated first. Further, the precipitate obtained from any given mixture in the less acid medium contained a lower percentage of lecithin, and therefore a higher percentage of caseinogen than the complex given by the same mixture in the more acid solution, as would indeed be expected from the results of the analyses of the precipitates obtained from the same lecithin-caseinogen mixture over a wide range of hydrogen ion concentration. In the mixtures richer in lecithin less of this substance was carried down in the complex than was the case with the corresponding mixtures in the more acid medium; on the other hand from the mixtures poorer in lecithin a relatively greater proportion was precipitated, but the effect of this on the final

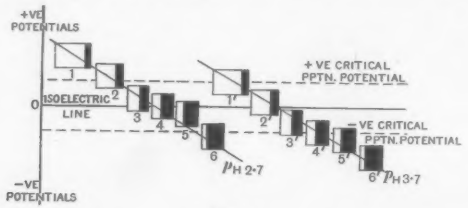

Fig. 4.

percentage of lecithin in the precipitate was more than counterbalanced by the much greater proportion of the added caseinogen that was also carried down. All these differences are readily explained when it is remembered that in the less acid solution a relatively much larger quantity of the now feebly charged caseinogen is required to reduce the high charge on the lecithin particles to the critical precipitation value.

Fig. 4 presents these relationships. Here the ordinates express the charge on the colloidal complex, positive or negative, as the case may be, the zero line indicating the completely discharged or isoelectric condition. The lines drawn parallel to and respectively above and below this represent the critical values of positive or negative charge which are just sufficient to keep the colloidal particles in permanent dispersion. In other words precipitation can occur only of particles carrying a charge whose magnitude is expressed by some point occurring between these two lines. The black portions of the rectangles are imagined as being proportional in area to the amounts of lecithin, and the white portions to the amounts of caseinogen, associated in the formation of complexes that may either be precipitated or remain in dispersion. It is not necessarily the case, nor indeed is it likely, that the

whole quantity of each substance added to a given mixture is used for the formation of such complexes; some of either constituent may remain free, but for the sake of simplicity of representation it is assumed that the whole of the lecithin and regularly progressing quantities of caseinogen are so combined. This assumption merely simplifies but does not invalidate our argument. The height of each rectangle is intended to express the (supposedly constant) range of variation of charge on the complex particles which it is regarded as containing. By following the figure from left to right it is easy to see how the complexes richest in caseinogen are devoid of particles whose charge is below the positive critical precipitation value (rectangle 1); how the first complex particles to be precipitated are still relatively rich in caseinogen (rectangle 2); and how it comes about that the greatest total amount of precipitate and of caseinogen carried down are associated with a complex whose caseinogen percentage is relatively smaller (rectangle 3). Finally by comparing rect-

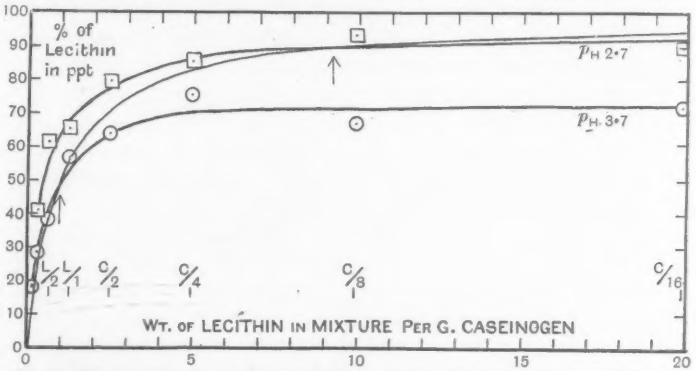
% of Lecithin in ppt
p_H 2·7
p_H 3·7
L/2 L/1 C/2 C/4 C/8 C/16
WT. OF LECITHIN IN MIXTURE PER G. CASEINOGEN
0 5 10 15 20
0 10 20 30 40 50 60 70 80 90 100

Fig. 5. The arrows indicate the compositions of the mixtures that are precipitated unchanged at p_H 2·7 and p_H 3·7 respectively.

angles 3, 4 and 5 it will be seen how complete precipitation of all the lecithin present may occur in complexes of different compositions, although only one of these (represented by rectangle 4) is really isoelectric, having the range of charge of its particles equally distributed about the zero line. Rectangle 6 represents the partial precipitation of a complex carrying a high negative charge on account of the presence of a large percentage of lecithin. The corresponding relationships for the less acid medium of p_H 3·7 are represented by rectangles 1′ to 6′ in which a much larger percentage of the now feebly charged caseinogen is represented as being necessary for the reduction of the high charge on the lecithin to the critical precipitation value.

We pass now to a consideration of Fig. 5 in which the changes of composition of the precipitated complex observed in these experiments are plotted, in terms of percentages of lecithin as ordinates, against the corresponding ratios in which the constituent substances were mixed expressed numerically (not logarithmically) as abscissae. From the resulting curves it is seen that after

the first rapid increase in the percentage of lecithin in the precipitate, further increase in the total amount of lecithin present in the solution produces less and less increase in the proportion of this substance in the precipitated complex. The occurrence of this long range over which the composition of the precipitated complex varies but little in response to very wide variations in the ratio in which its constituents are mixed readily accounts for the tendency on the part of some authors to regard this complex as a definite chemical compound, the small variations in the composition of the actual precipitate being ascribed to admixture of small and variable quantities of its constituents. But our observations show that this range of almost constant composition is but part of the continuous and in other ranges more pronounced variation in the composition of the complex that would be expected on purely colloid-chemical grounds.

SUMMARY.

1. The percentage of lecithin in the precipitates obtained by the addition of a mixture of lecithin and caseinogen to buffer solutions of graded acidity increases with increasing hydrogen ion concentration of the medium.

2. When mixtures of caseinogen and progressively increasing quantities of lecithin are added to the same buffer solution the increase, at first rapid, in the percentage of lipin in the several precipitates becomes more and more gradual until a stage is reached at which very considerable further increase of the total proportion of lecithin added produces but slight increase in the percentage of this constituent in the precipitated complex.

3. These results are explained in terms of the mutual precipitation of two colloids whose opposite charges vary in intensity with the hydrogen ion concentration of the dispersion medium.

I wish to take this opportunity of expressing my indebtedness to Mrs K. Pinhey for valuable help with these experiments.

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CIII. STRUCTURE AND ENZYME REACTIONS. PARTS V AND VI.

THE SYSTEMS GLUCOSE-ENZYME AND ESTER-CATALYST.

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In the introduction to Part I of this series [Przylecki *et al.* 1927] it was shown that the introduction of an adsorbent into certain enzyme-substrate systems diminishes the velocity of reaction in those cases where both enzyme and substrate undergo adsorption. As was shown in Part III [Przylecki and Niedzwiecka, 1928] retardation of reaction may also be observed in systems where the presence of an adsorbent leads to a reduction in the number of collisions taking place in unit time between enzyme and substrate particles. Similarly, it might be conceived that insufficient stirring or agitation of the suspension of adsorbent on which either the enzyme or the substrate is adsorbed could reduce the velocity of reaction. As was shown in Part I, this phenomenon is not of general occurrence and depends upon the velocity of reaction and rate of diffusion for each particular case; where the latter factor is negligible in comparison to the former, no retardation should be observed.

The previous papers of this series deal with practically irreversible reactions, whose velocity is proportional to the concentrations of enzyme and substrate, whilst the products of reaction are adsorbed to a considerably less extent than the substrate. The latter factor, while of no great importance in the case of irreversible reactions, may become of importance in those cases where only the products of reaction are adsorbed. In such cases, the presence of an adsorbent may lead to an acceleration in the velocity of the later stages of an enzyme reaction, since the velocity constant may be expressed

$$K = \frac{[\text{enzyme-reaction products}]}{[\text{enzyme}]\,[\text{reaction products}]}.$$

Thus a part of the products of reaction is separated by adsorption from chemical combination with the enzyme, leaving the latter free to combine with the substrate.

An adsorbent might therefore be chosen such that it not only does not retard, but even accelerates, irreversible reactions, and this acceleration will be greater the greater the value of K, *i.e.* the greater is the concentration of the products of degradation and the smaller that of the initial substance.

In Part I the possibility was raised of the acceleration of reversible reactions and of the shifting of the equilibrium point under the influence of an added adsorbent. This might occur in those cases where either the products of synthesis or of degradation are differentially adsorbed. The following conclusion, based on the law of mass action, might hence be drawn, only those cases being considered in which the adsorption of the substrates is reversible, and in which the adsorbent does not enter into chemical reaction with the substrates.

The kinetics of enzyme reactions would here depend upon two factors—the shifting of the point of equilibrium and the degree of adsorption. Where the degrees of adsorption of substrates and products are similar, both when the products of synthesis or of degradation are present in excess, the velocity of reaction diminishes, as a result of the removal by adsorption of the substrates from the sphere of action of the enzyme. If, however, the products of synthesis are adsorbed to a greater extent than the substrates the reaction will depend kinetically upon its relation at any given moment to the definitive equilibrium point, *i.e.* upon the quotient

$$K = \frac{[\text{products of degradation}]}{[\text{products of synthesis}]}.$$

Where the products of synthesis are present in excess, so that the reaction is proceeding in the direction of degradation, the addition of an adsorbent should retard the reaction, as in the case of the system amylase-charcoal-polysaccharide (Part II). Should, however, the reaction of synthesis be dominant several possibilities might arise.

If the substrates of the reaction of synthesis do not undergo adsorption, whilst at the same time the adsorbed enzyme retains its activity, the reaction should be accelerated almost from the commencement, since the products of synthesis are being continually removed by adsorption from the reaction mixture. The difference between reaction in a homogeneous and a heterogeneous system will be greater the closer the former system is to its equilibrium point, and the quantity of synthesised product present at any one moment in the former system after the attainment of equilibrium will be smaller than in the latter. In such a case, the value of K for the system as a whole diminishes with increasing adsorption of the synthesised products, although in the disperse phase alone it will not have undergone any change, except that the concentrations of all the constituents will be smaller.

In those systems, however, in which both the substrates and synthesised bodies undergo adsorption, but the latter to a greater extent than the former, conditions are more involved. Whilst, in the above case, the velocity of the reaction of synthesis is almost from the beginning greater in the presence than in the absence of an adsorbent, in the present case two stages of the reaction may be distinguished. In the first stage, the concentration of substrates in solution is, as a result of their adsorption, smaller than in a homogeneous system, whilst the adsorption of products of synthesis is too small noticeably

to affect the velocity of reaction. As a result, the addition of an adsorbent to a system at this stage may retard the reaction of synthesis. The second stage supervenes when the reaction is approaching equilibrium; here adsorption of the substrates is insignificant, but that of the synthesised products is so large as to cause acceleration of the reaction. Graphically, we would here obtain two curves—one rising steeply but soon becoming flatter, and the second at first less steep, but retaining its initial slope longer, so that eventually it cuts the former curve and rises above it. This reasoning applies, of course, only to reactions for which the velocity of reaction is a function of the concentration of the substrates. In a system in which the latter are present in excess, or are very feebly adsorbed, the adsorption of a part of them need have no perceptible effect upon the velocity of reaction in its initial stages. The kinetics of such a reaction would be unaffected by the addition of an adsorbent.

In the case of certain biological syntheses, the application of the above illustrations may be exceedingly involved. Thus, for such heterogeneous reactions as the synthesis of glycogen where the eventual product of synthesis undergoes adsorption, and the products of the various stages of synthesis are located in different, spatially separated parts of the cell, the velocity of reaction would be diminished by the presence of an adsorbent. This, however, need not apply to a reaction of synthesis catalysed by a number of enzymes, in which the whole process takes place in one part of the cell, whilst the intermediate products are relatively not adsorbed to any great extent. Whether the synthesis of polysaccharides takes place under such conditions it is not yet possible to decide, as our knowledge of this process is insufficient. The biological synthesis of fats is, however, in all probability of this type. Proteins are possibly synthesised in a system of the first type, in which the reaction proceeds in several stages, spatially separated from each other. Little consideration has, however, up to the present been given to this problem.

A third general possibility is of the adsorption of the products of degradation being greater than that of the substrates. In such cases, in which the synthesised body does not undergo adsorption and is present in excess, the addition of an adsorbent should accelerate the reaction of degradation almost from the beginning, whilst, where the product of synthesis undergoes adsorption, parallel conditions obtain to those found in certain systems of the second type, *i.e.* the velocity of degradation would be retarded in its initial and accelerated in its final stages. In systems of this type, the velocity of synthesis should be diminished by the addition of an adsorbent, which would lower the concentration of substrates.

Thus the addition of an adsorbent to systems of the type enzyme-products of synthesis-products of degradation, in which the enzyme acts practically reversibly, may cause the following changes.

1. *In the equilibrium point.* K remains unaltered in the disperse phase, but in the system as a whole, including those of its constituents present in the

adsorbed condition, it is either changed in favour of the reaction of synthesis, should the products of the latter be more strongly adsorbed than the substrates, or in favour of the reaction of degradation, should the contrary be the case.

2. *In the velocity of reaction.* This may remain unaltered, be retarded or be accelerated.

The influence of the adsorbent on these two factors, K and velocity of reaction, has been expressed mathematically by Sym, as follows.

If a be the concentration of the reacting substance in the absence of an absorbent, then after the addition of the latter the concentrations in solution and on the adsorbent will be together $a_1 + \frac{a}{m} a_1^{1/n}$, whilst at some time during the course of the reaction, they will be

$$a_1 - x + a/m\,(a_1 - x)^{1/n} + x_1 + \beta/m\,.x'^{1/p},$$

and the quantity of substance y which has entered into reaction would be given by the equations

$$y = x' + \beta/m x'^{1/p} = x + a/m\,[a_1'^{1/n} - (a_1 - x)^{1/n}].$$

The velocities of reaction and the equilibrium constants would then be as follows, for systems in which adsorption is reversible and mutually independent for each constituent.

1. In the absence of an adsorbent,

$$\frac{dy}{dt} = k_1(a - y) - k_2 y, \text{ and } K_1 = \frac{a - y}{y_1}.$$

2. Adsorption of the reacting substance,

$$\frac{dy}{dt} = k_1(a_1 - x) - k_2 y, \text{ and } K_1 = \frac{a - x}{y}.$$

$$K_2 = \frac{(a_1 - x_1) + a/m\,(a_1 - x_1)^{1/n}}{y_1}.$$

3. Adsorption of product of synthesis,

$$\frac{dy}{dt} = k_1(a - y) - k_2 x', \text{ and } K_1 = \frac{a - y_1}{x_1},$$

$$K_3 = \frac{a - y_1}{x_1' + \beta/m\,.x_1'^{1/p}}.$$

4. Adsorption of both substances,

$$\frac{dy}{dt} = k_1(a_1 - x) - k_2 x', \text{ and } K_1 = \frac{a_1 - x_1}{x_1'},$$

$$K_4 = \frac{a_1 - x_1 + a/m\,(a_1 - x_1)^{1/n}}{x_1' + \beta/m\,.x_1'^{1/p}}.$$

In view of the great number of enzymic reactions which take place in the living organism, often reversible, and whose substrates, products or enzymes are adsorbed by substances found within the cell, the above theoretical reasoning calls for experimental verification. A number of researches with this end in view are now being conducted at this laboratory; in this paper the results obtained for two systems will be described. These are the systems

enzyme-glucose-charcoal and hydrochloric acid-aliphatic acid-alcohol and ester-charcoal.

In the first system we have the reaction:

$$\text{enzyme} + \text{conc. glucose solution} \rightleftharpoons \text{enzyme} + \text{hexobiose} + H_2O.$$

The reaction attains a definite equilibrium point the value of which, in systems without charcoal, depends upon the initial concentration of glucose. This system can be used for the demonstration of the postulate that structure may influence reaction by changing the value of K and so augmenting synthesis, since the hexobiose formed is considerably more adsorbed than glucose, in solutions containing over 20 % of the latter and from 1 to 2 % of maltose.

The reaction, acid + alcohol $\rightleftharpoons$ ester + water, proceeds under the catalytic influence of hydrochloric acid in a medium containing excess of alcohol. The addition to this system of preparations of charcoal, which adsorbs the organic acid to a considerable extent, but not the ester, brings about the acceleration of the reaction of hydrolysis.

The systems charcoal-enzyme-glucose and enzyme-glucose.

The work of Hill [1898], Emmerling [1901], Fischer and Armstrong [1902] and Bourquelot *et al.* [1913] has established that if an appropriate enzyme be added to a concentrated glucose solution, a reaction of synthesis of disaccharide proceeds to a certain equilibrium point.

This system has been selected as suitable for the experimental establishment of the hypothesis that the equilibrium point of reversible reactions may be shifted in favour of synthesis by the addition of a substance on which the products of synthesis are adsorbed to a greater extent than are the substrates.

A fundamental condition for this is the almost quantitative adsorption of the enzyme. Whilst from theoretical considerations it follows that the ratio of glucose to maltose in solution would be changed in favour of the former, a consideration of adsorbed glucose and maltose should indicate augmented synthesis of the latter.

A number of preliminary experiments had first to be undertaken:

(1) with a view to establishing that glucose and maltose are adsorbed by charcoal in concentrations approaching those which are obtained in the enzymic synthesis of maltose;

(2) for the analysis of these two sugars in adsorption on charcoal;

(3) in order to carry out a reaction lasting several months under sterile conditions, without the use of antiseptics, which would affect the relative adsorption of the sugars, and diminish their absolute adsorption;

(4) in order to elaborate a method for the quantitative determination of maltose in the presence of a large excess of glucose.

The researches of Bourquelot, of Emmerling and of Hill were of great assistance in the execution of our research. From the papers of these authors were taken the concentration of glucose used, the time of reaction, and the selection of the enzyme, as well as the method for the estimation of small quantities of disaccharide in the presence of a large excess of glucose.

A. *The equilibrium of the system enzyme-glucose.*

Our first experiments had as their objects the establishment of the necessary technique for working over long periods of time without the use of disinfectants; further, the achievement of conditions relatively close to those of final equilibrium in the absence of charcoal, and the determination of the degrees of adsorption and of elution, together with the conditions under which the latter takes place.

(*a*) *Sterilisation.* The elutive effect of ordinary antiseptics militated against their use in these experiments, and the following technique was therefore used for the maintenance of aseptic conditions. The glucose solutions, with or without charcoal, were sterilised by heating in an autoclave for 30 minutes in 250 cc. Erlenmeyer flasks stoppered with cotton-wool. The enzyme solutions were sterilised either by passage through a Chamberland filter or by the addition of powerful antiseptics such as ethyl alcohol, which were then removed under diminished pressure at 20°. The sterilised enzyme solutions were in some cases measured out from sterilised graduated cylinders and then added to the sterilised glucose solutions. In others, the flask containing the glucose solution was tightly stoppered with cotton-wool, through which passed two tubes, one connected to a filter pump and the other to a graduated vessel containing sterilised enzyme solution. The diminution in volume of the contents of the latter vessel represented the volume of enzyme solution added to the glucose solution. In many cases, as an additional precaution against infection, methyl alcohol was added. This is a fairly strong antiseptic, whilst at the same time its elutive power is inconsiderable.

The above measures enabled us to maintain sterile conditions for over 6–7 months. Before each determination, the solutions were tested for sterility, and only those from which micro-organisms were absent were taken for analysis.

(*b*) *Choice of enzyme.* Emmerling [1901] showed that isomaltose and dextrin are produced in concentrated solutions of glucose in the presence of yeast maltase, whilst Bourquelot *et al.* [1913] used several enzymes such as emulsin for the synthesis of disaccharides. In this research, yeast maltase prepared according to Emmerling was used.

(*c*) *Determination of synthesised disaccharide.* The method described by Emmerling was applied, with slight modifications. Glucose was completely decomposed by fermentation, and the remaining disaccharide hydrolysed by hydrochloric acid, the glucose thus formed being then determined by Bertrand's method. The fermentation was carried out by the agency of *Saccharomyces marxianus, S. exiguus* or *Willia anomala.*

(*d*) *Establishment of equilibrium.* Two points had here to be proved; firstly that the velocity of synthesis is almost unaffected by the addition of charcoal at the initial stages of the reaction, and secondly that the high degree of adsorption of maltose on charcoal shifts the equilibrium point of the system as a whole in the direction of synthesis. For the establishment of these facts absolute equilibrium was not essential, conditions close to this state being sufficient. Since solutions from which samples had been taken for analysis could not be used further, and as the entire contents of a flask had to be used for the determination of the degree of elution, the attainment of conditions close to equilibrium had to be judged by the determination in different flasks of the ratio of glucose to maltose at fortnightly intervals beginning a month after the commencement of the experiment. In this way, it was found that in the absence of charcoal initially 20 % glucose solutions contained at equilibrium 1·48 % of disaccharide (see Table I).

Table I. *Synthesis of hexobiose.*

Initial concentration of glucose (%)	Hexobiose found after 5 months (%)
20	1·29, 1·58, 1·71, 1·34 Mean 1·48

B. *Adsorption of glucose and disaccharide on charcoal.*

Having determined equilibrium conditions in the absence of charcoal the system enzyme-20 % glucose could now be studied in its presence.

In order to obtain measurable differences in the equilibrium point of the reaction it was necessary to create conditions such that the quantity of adsorbed disaccharide was as great as practicable. This was done by introducing large quantities of adsorbent, the components of the systems examined being present in the following quantities: 5 g. of charcoal, 50–100 cc. of a solution containing 10–20 % of glucose and 1–2 % of maltose. The charcoal preparations used were either of local manufacture or Merck's "Carbo medicinalis." The mixtures were placed in a mechanical shaker for 4–6 hours, after which they were left in a thermostat at 37° for 12 hours, and then filtered. The results obtained are given in Tables II, III and IV. The values found show that barely 7·2 % of glucose is adsorbed on local charcoal from a 16·6 % solution, and 8·1 % from a 9·0 % solution. Maltose is adsorbed far more intensely, to the extent of 63·5 % from a 0·86 % solution, and 43·2 % from a 1·4 % solution. Considerably higher degrees of adsorption were observed for Carbo medicinalis, being 12 % from a 20 % solution of glucose and 97 % from a 2 % solution of maltose.

From the point of view of our experiments, it was of great importance to establish the degrees of adsorption of glucose and maltose in systems containing originally only the former sugar, to which maltose solution is added later. Such conditions would correspond to those obtaining during the enzymic synthesis of hexobiose in the presence of an adsorbent.

Table II. *Adsorption and elution of maltose and glucose.*

Percentage concentrations.

	Without charcoal	With charcoal: Before elution: In solution	With charcoal: Before elution: % adsorption	With charcoal: After elution: In solution	With charcoal: After elution: % adsorption
Glucose solution	9·0	8·29	8·1	9·0	0
Maltose solution	0·86	0·31	63·5	0·70	18·6

Table III. *Adsorption of glucose and maltose separately and together.*

Percentage concentrations.

Solution	Without charcoal	With charcoal	% adsorption
Glucose	16·6	15·4	7·2
Maltose	1·4	—	43·2
Maltose 1·4 %, with glucose 16·6 %	—	—	33·7

90 cc. of 22 % glucose were added to 5 g. of charcoal, the whole was shaken 3 hours and left overnight. 10 cc. of 2 % maltose solution were then added drop by drop and with constant shaking, an interval of one hour being allowed after the addition of each 2 cc. The results obtained are given in Tables III and IV.

Table IV. *Adsorption and elution of glucose and maltose together and separately.*

Percentage concentrations.

Solution	Without charcoal	With charcoal: Before elution: % in solution	With charcoal: Before elution: % adsorption	With charcoal: After elution: % in solution	With charcoal: After elution: % adsorption
Glucose	20·11	17·7	12·0	19·3	4·0
Maltose	1·97	0·058	97·0	0·97	50·0
Maltose and glucose together		0·674	65·8	1·50	24

These values are based upon analyses and calculations of the concentrations of solutions of maltose with glucose with and without charcoal. Maltose was determined after the fermentative destruction of glucose by hydrolysis in the presence of hydrochloric acid, the glucose thus formed being then determined by Bertrand's method. The amount of adsorption of glucose was determined polarimetrically and was calculated from the difference between the optical rotations with and without adsorbent of the solution of maltose and glucose less that of the maltose alone. Comparative values are given only for the adsorption of maltose, which alone concerns us in the study of equilibrium conditions.

The adsorption of maltose in the presence of 16·6 % glucose on charcoal preparation No. 1 (Table III) is considerably smaller than in its absence. Thus adsorption is here diminished by 22·2 %, from 43·2 to 33·7 %. The

second charcoal preparation which normally adsorbs 97 % of maltose from solution exhibits an adsorption of maltose of only 66 % in the presence of 20 % glucose, a diminution of 31 %.

A number of experiments, the detailed results of which will not here be given, showed that the diminution of adsorption of maltose caused by the addition of glucose depends upon the concentrations of these sugars. Thus the greater the concentration of maltose used the more closely do the adsorptions of glucose and maltose separately approach each other, and the same is the case when the concentration of glucose is lowered. On the other hand, the difference in the degree of adsorption of maltose in the presence of glucose is intensified as the concentration of the former sugar rises, and this difference becomes even more accentuated in more concentrated solutions of glucose. These considerations possess a certain biological significance. Thus, changes in the enzymic equilibrium of this reaction will, up to a certain point, be the more marked the greater the relative concentration of glucose, and the augmented yield of biose due to the presence of an adsorbent will vary inversely as the concentration of maltose in equilibrium in its absence. These conclusions follow from the very similar degrees of adsorption of these two sugars. The attainment of the differential adsorption of glucose and maltose thus depends upon their concentrations being chosen so as to fall in different regions of the adsorption isotherms.

The above would be of considerably less importance in those cases in which the degree of adsorption of the synthesised body differs greatly from that of its progenitors, as in the case of glycogen and glucose, where glucose undergoes only negligible adsorption upon protein whilst that of glycogen is very considerable. In this case, the presence of an adsorbent may accelerate synthesis and change the equilibrium constant even with large concentrations of glycogen and small concentrations of glucose.

C. *Elution of the sugars.*

Changes in the equilibrium point of this enzymic reaction are apparent only if the adsorbed biose be taken into consideration. Thus, for the determination of K it was necessary to find the sum of glucose and maltose adsorbed and in solution. This was done by elution of adsorbed sugars by means of amyl alcohol, which had been shown by Przylecki and Niedzwiecka [1928] to act as a powerful elutive agent of uric acid from charcoal. The procedure was as follows. Solutions of glucose and maltose separately or together were added to charcoal in the same order and applying the same precautions as described previously. Amyl alcohol was then added to the mixture, which was then mechanically shaken for 3–6 hours, left tightly stoppered for 12 hours, and again shaken for 3–6 hours. The mixture was then filtered into separating funnels, and the aqueous layer of the filtrate taken for analysis. The optical rotation of the solution of sugars, either mixed or separate, was now measured,

and the same analytical procedure applied as before to the determination of the concentrations of the individual sugars.

The determination of maltose in the presence of excess glucose and of amyl alcohol was with certain modifications effected according to Emmerling. In order to establish that the presence of amyl alcohol did not in any way influence the reaction of fermentation, the following experiments were carried out. Yeast maltase was added to the above filtrates, containing glucose and maltose separately or together. The mixtures were then heated, to drive off amyl alcohol and to destroy the hexobiose-synthesising enzyme, and the excess glucose was destroyed by fermentation.

The results obtained showed that no reducing bodies remained after the fermentation of glucose, and that when maltose was present the content of this substance did not diminish under these conditions. Emmerling's method is therefore applicable to this analysis.

The results given in Tables II and IV show that for the sugars taken separately glucose undergoes almost quantitative elution, whilst maltose is only partially eluted, 18·6 to 50 % of the total quantity remaining in adsorption. Where a mixture of maltose and glucose is taken, a far smaller quantity of the former remains adsorbed after the addition of amyl alcohol, the percentage adsorption falling from 65 to 24 %.

D. *Comparison of enzymic equilibrium in the presence and in the absence of charcoal.*

The technique employed in these experiments was similar to that used in the preliminary investigations. 50–100 cc. of 20 % glucose solution containing yeast maltase was added to 5 g. of charcoal, and analyses were made of the sugars after 1–2 weeks and 2–6 months. The relative concentrations of glucose and hexobiose were determined separately for the solution and for the whole system after elution with amyl alcohol. Glucose was determined either polarimetrically or by Bertrand's method as the difference between the total amount of glucose found after acid hydrolysis of the whole mixture and that produced by hydrolysis of the biose alone, which, as before, was isolated by fermentation of glucose.

Table V. *Synthesis of hexobiose in the presence of 5 g. of charcoal to 50–100 cc. of 20 % glucose.*

No. of determinations	Time	Volume of solution cc.	Without charcoal	With charcoal		Hexobiose after elution
				In solution	Reversibly adsorbed	
5	3–7 days	50	0·250	—	—	0·270
4	„	100	0·304	—	—	0·310
7	2 months	50	1·40	1·34	0·688	2·03
8	5 „	—	1·67	1·61	0·81	2·42

The results given in Table V afford a satisfactory experimental confirmation of the postulates put forward in the theoretical part of this paper. During

the first stages of the reaction, lasting 3–7 days, the presence of charcoal has no appreciable effect upon the velocity of the reaction of synthesis. Neither in the solution alone, nor in the whole system can any great difference be seen in the yield of synthesised hexobiose. If anything, it would appear that the amount of hexobiose synthesised in the presence of charcoal is smaller than in its absence, although this difference is so small as hardly to exceed the limits of experimental error.

This phenomenon appears to be due to the fact that at this stage the reaction is still far from its end-point, and its velocity should not therefore be greatly affected by the adsorption of the small quantities of hexobiose present. Similarly the removal by adsorption of glucose from solution, leading to a diminution in its concentration of from 18·6 to 20 % is unable greatly to diminish the velocity of the reaction of synthesis. As at this stage the very small quantities of hexobiose produced render its determination difficult, only its total amount in solution and adsorption was determined.

The results obtained after a lapse of 2–5 months are entirely different. Here very considerable differences exist between the amounts of hexobiose synthesised in the presence or absence of charcoal. Whilst in the latter case 1·40 % of biose is found after 2 months and 1·67 % after 5 months, in the presence of charcoal we find 2·03 and 2·42 % after 2 and 5 months respectively. This represents in the absence of an adsorbent a yield of 6·0 and 8·35 % respectively on the glucose used, whilst in the presence of charcoal the yields are 10·1 and 12·1 % respectively. The actual difference is undoubtedly even greater, since the uneluted hexobiose has not here been taken into consideration.

No attempt has been made to express the above differences in terms of K, since we have no certainty that definitive equilibrium has been attained—indeed, in those systems examined after 2 months there can be no doubt that equilibrium had not been reached, and for this reason the differences are expressed only as percentages. Thus, after 2 months the presence of charcoal increased the yield of hexobiose by 45 % and after 5 months by 44·1 %.

As to the solutions alone, the concentrations of biose are substantially the same whether an adsorbent is present or not. The cause of augmented synthesis lies solely in the charcoal, on which 0·688 % of the biose was adsorbed after 2 months, and 0·81 % after 5 months, corresponding respectively to 34·4 and 33·8 % of the total quantity synthesised.

Influence of adsorbent upon reactions of esterification.

The effect produced by the addition of adsorbent charcoal (Merck's) to systems containing alcohols, aliphatic acids and their esters, and hydrochloric acid as catalyst, was investigated.

The acid content was determined by titration with barium hydroxide solution, using phenolphthalein as indicator, and esters were determined by

hydrolysis with excess sodium hydroxide solution and back-titration with standard acid.

The procedure generally applied was as follows. Two flasks containing an aqueous solution of alcohol (solution III) of known concentration were taken, and a known quantity of aliphatic acid or ester was added to one flask (solution I). To the other a known quantity of hydrochloric acid was added (solution II); the concentration of alcohol in both flasks was kept equal.

After these solutions had been warmed to the reaction temperature of 37° the concentrations of their constituents was determined. Equal volumes of solutions I and II were then mixed, and the resulting solution IV analysed. The concentration of the constituents of solution IV amounted in every case to half the sum of those of solutions I and II separately. The time at which solutions I and II were mixed was noted, and represents the time of commencement of reaction.

To a portion of solution IV adsorbent charcoal was added immediately after mixing, and, after violent shaking over a short period, the degree of adsorption was determined in this mixture, VII. The degree of adsorption of the individual constituents of the latter mixture was determined by adding adsorbent charcoal to solutions prepared by mixing equal volumes of solutions I and III (solution V) or II and III (solution VI) and analysing after shaking. The sum of the quantity of compounds adsorbed from solutions V and VI was always close to that adsorbed from solution VII.

The concentrations of the substrates of solutions IV and VII were determined at various intervals of time up to the attainment of equilibrium, after which the degree of adsorption of the substrates and reaction products was determined.

10 cc. of solution was in every case taken for titration.

Results.

I. *The system glycerol-butyric acid-charcoal-catalyst.*

Applying the same numeration as above, the following solutions were prepared:

I. 400 cc. of glycerol (sp. gr. 1·233) + 350 cc. water + 50 cc. butyric acid.
II. 400 cc. „ „ + 388 cc. „ + 12 cc. conc. HCl.
III. 400 cc. „ „ + 400 cc. „

The concentration of acid in solution I was equivalent to 336·9 cc. and in II to 88·6 cc. of 0·05 *N* barium hydroxide, half of the sum of which amounts to 212·7 cc., whereas that of solution IV was 212·3 cc. The concentrations of solutions V and VI were respectively 168·2 and 44·4 cc., after adsorption 137·5 and 44·4 cc. respectively, a total of 181·9 cc.

The concentration of hydrochloric acid in VI is thus unaffected by the addition of 2 g. of adsorbent per 100 cc. of solution. It was noticed incidentally that the filtrate from VI after the addition of charcoal gave a preci-

pitate of barium sulphate on the addition of barium hydroxide. Solution VII contained 182·2 cc. of 0·05 *N* acid, or only 0·3 cc. more than solutions V and VI together.

Table VI. *cc. of* 0·05 *N butyric acid or ester.*

Date ...	6. xii	7. xii	15. xii		16. xii		18. xii		$K = \frac{[\text{acid}]}{[\text{ester}]}$
	Acid	Acid	Acid	Ester	Acid	Ester	Acid	Ester	
Without adsorbent	167·9	133·3	107·3	60·6	106·5	61·4	106·2	61·7	1·72
With adsorbent: in solution	137·8	112·0	90·2	53·6	89·6	54·1	89·6	54·1	1·66
With adsorbent: in adsorption	30·1	26·1	21·0	2·8	21·3	2·9	21·3	2·9	—
Total content	167·9	138·1	111·2	56·4	110·9	57·0	110·9	57·0	1·97

The results are given in Table VI. The quantity of adsorbed butyric acid was determined by direct titration of the residue left after filtering 200 cc. of solution VII, and deducting from the value obtained the quantity of acid contained in the solution absorbed by the filter-paper and between the grains of charcoal. Filtration of solution VII before the addition of charcoal showed that the filter-paper retains 5·6 cc. of 0·05 *N* acid, and it may be taken that the second item amounts to about 4 cc. of solution or about 36·0 cc. of 0·05 *N* acid. Since the filter-paper with residue after the filtration of 200 cc. of solution contains the equivalent of 469·0 cc. of 0·05 *N* acid, and the correction is altogether 41·6 cc., the figure of 427·4/20 = 21·3 cc. 0·05 *N* butyric acid is arrived at for the adsorption of this acid upon charcoal. The amount of adsorption of the ester is arrived at by subtracting from the difference between the acid + ester content with and without charcoal, namely

$$167{\cdot}9 - 143{\cdot}7 \text{ cc.} = 24{\cdot}2 \text{ cc. } 0{\cdot}05\ N \text{ acid + ester},$$

representing adsorption of butyric acid and of its glyceride, the above value of 21·3 cc. found for acid alone, leaving a value of 2·9 cc. of 0·05 *N* ester adsorbed. Equilibrium in the heterogeneous system is thus represerted by $K = 1{\cdot}97$, as against a mean value of 1·69 for the dispersing medium alone. The introduction of an adsorbent into this system increases, therefore, the equilibrium constant, which is changed in favour of hydrolysis.

II. *The hydrolysis of ethyl acetate.*

The procedure adopted for this system was identical with that of the preceding, except that the substrates were ethyl acetate dissolved in 44·4 % alcohol. The quantity of charcoal used here was 3 g. per 100 cc. of solution, and as before, 10 cc. of solution were in all cases taken for titration, using 0·0397 *N* barium hydroxide. Using the same numeration of solutions as previously, solutions IV and VII contained initially 93·5 and 82·5 cc. of 0·0397 *N* ester, and 7·9 and 7·5 cc. of 0·0397 *N* hydrochloric acid respectively. Equilibrium was attained in solution IV in 13 days, the initial and final conditions being illustrated in Table VII.

Table VII. *cc. of 0·0397 N acetic acid or ester.*

	Initial content of		Final content of		$K = \frac{[\text{acid}]}{[\text{ester}]}$
	Acid	Ester	Acid	Ester	
Without adsorbent	0	93·5	47·5	46·0	1·03
With adsorbent { in solution	0	82·5	43·2	41·3	1·02
With adsorbent { in adsorption	0	11·0	1·7	7·5	—
Total content	0	93·5	44·9	48·8	0·92

The degree of adsorption of the compounds present in the equilibrium mixture is derived from the values found for each constituent separately at concentrations close to those found at equilibrium. Thus 7·4 cc. of 0·0397 *N* ethyl acetate were adsorbed from a solution containing 40·9 cc. Assuming that a certain proportionality exists between the amounts in adsorption and in solution for small changes in concentration, it is here taken that 7·5 cc. are adsorbed from a solution containing 41·3 cc. of 0·0397 *N* ester. Similarly for acetic acid 2·3 cc. were adsorbed from a solution containing 57·9 cc., whence 1·7 cc. would be adsorbed from the equilibrium mixture containing 43·7 cc. of 0·0397 *N* acetic acid. These calculated values are in satisfactory agreement with experimental values, as is shown by a comparison between the sum of adsorbed and unadsorbed acid and ester found in solution VII, and the value for the amount of ester present at the beginning of the reaction, namely the equivalent of 93·7 and 93·5 cc. of 0·0397 *N* barium hydroxide respectively.

In this case the degrees of adsorption of the acid and ester are different from those existing for the first reaction studied, and the equilibrium constant is therefore lowered by the addition of an adsorbent, *i.e.* the reaction of hydrolysis does not proceed as far in the presence as in the absence of an adsorbent. At the same time it will be seen that the equilibrium constant remains unaffected in the dispersing medium.

III. *The synthesis of ethyl acetate.*

This system consisted of 12·4 % ethyl alcohol containing acetic acid, with hydrochloric acid as catalyst; 5 g. of charcoal per 100 cc. of solution were added. The results, expressed in terms of cc. of 0·0397 *N* barium hydroxide are given in Table VIII.

Table VIII. *cc. of 0·0397 N ethyl acetate or acetic acid.*

Date ...	2. iv HCl	2. iv Acid	3. iv Acid	4. iv Acid	5. iv Acid	6. iv Acid	16. iv Acid	18. iv Acid	18. iv Ester	$K = \frac{[\text{acid}]}{[\text{ester}]}$
Without adsorbent	6·7	56·0	49·4	48·4	48·2	48·0	47·8	47·8	8·2	5·8
With adsorbent { in solution	5·0	46·5	43·6	41·3	38·8	36·2	34·7	34·0	6·1	5·6
With adsorbent { in adsorption	—	9·5	9·0	8·4	7·9	7·6	7·4	7·4	9·2	—
Total content		56·0	52·6	49·7	46·7	43·8	42·1	41·4	15·3	2·7

In this case a very considerable enhancement of synthesis has taken place as a result of the addition of charcoal. This is due entirely to adsorption, since the equilibrium attained in the dispersing phase ($K = 5·6$) is little

different from that of the homogeneous system, for which K is 5·8. In the system as a whole, however, including that part of its constituents which is in adsorption, 7·1 cc. more of ester have been synthesised, representing an increase in yield of over 86 %, with a consequent diminution of K to 2·7. Curves illustrating this system are given in Fig. 1. It will be seen that synthesis, although at first more rapid in the absence of an adsorbent, soon slows down, and that the first portion of Curve II, representing reaction in the presence of charcoal, is practically a straight line. This would be due to

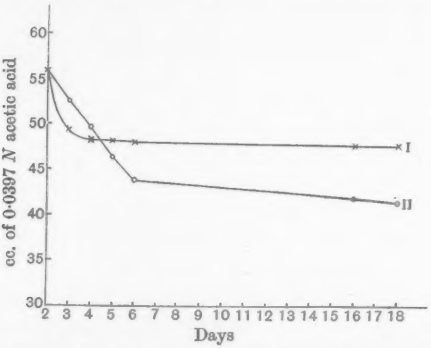

Fig. 1. Curves expressing concentration of acid in the absence (I) and presence (II) of adsorbent.

the practical absence of ester in the first stages of the reaction, which would only commence to exhibit retardation when the charcoal surface is under the given conditions close to saturation with ethyl acetate. The smaller initial velocity of reaction in the presence of charcoal is probably due to adsorption of acetic acid, as a result of which its active concentration is diminished.

The above three cases fully confirm the theoretical reasoning put forward in the first part of this paper and afford excellent examples of the mechanism of reversible reactions in the presence of adsorbents.

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CIV. THE AMINO-ACIDS OF FLESH.

II. COMPARISON OF THE DIAMINO-ACID CONTENT OF SOME NORMAL AND PATHOLOGICAL TISSUES.

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In a previous paper [Rosedale, 1922] the diamino-acid contents of the muscle of various farm animals were compared. Very slight differences could be detected between the tissues analysed, although it was pointed out that available methods of analysis did not ensure complete accord even when duplicates were made upon the same hydrolysate. During the intervening years, fresh endeavours have been made to secure greater accuracy in the analysis of proteins. Plimmer [1924] studied more closely the quantitative reaction of the hexone bases with nitrous acid. Plimmer and Phillips [1924] estimated histidine and tyrosine, after hydrolysis, by bromination. Plimmer and Rosedale [1925] have investigated each step in the Van Slyke estimation method and published certain modifications which secure greater ease and accuracy in the procedure. More recently, Plimmer and Lowndes [1927] have devised a method by which cystine may be estimated in the diamino-fraction of the Van Slyke method. The work now presented was undertaken as an extension of the data previously obtained. With two exceptions, muscle had not been analysed from different parts of the same animal. It has also been thought desirable to compare the results of the Van Slyke method with the older procedure of Kossel. The latter method which includes so many manipulations cannot necessarily claim greater accuracy than other methods of estimation, although in the hands of Kossel and Osborne the results have proved sufficiently concordant to form the basis of our knowledge of the subject to-day. Certain modifications of Kossel's original procedure have been introduced which avoid some of the difficulties. The opportunity has been taken of comparing certain pathological with normal tissues, and of analysing the muscle tissue of animals which had been fed upon known proteins.

Experimental.

After removing as much fat as possible the tissues were minced. They were then extracted in 2 l. of boiling water containing 2 cc. glacial acetic acid in order to coagulate the protein and to separate it from extractives. The liquid was poured off and the tissue squeezed dry in a cloth. The procedure of extracting and drying was repeated twice. The tissues were then

digested for 8–12 days at 37° with 1 g. of pepsin in 2 l. of 0·1 *N* HCl. Indigestible matter was filtered off and the total nitrogen estimated by Kjeldahl's method. The filtrate was then hydrolysed with 25 % HCl for 36 hours, when it was evaporated to dryness *in vacuo* once or twice to remove as much HCl as possible. The hydrolysate was finally brought to such volume that 100 cc. represented approximately 0·5 g. of nitrogen. Portions of 100 cc. were then analysed by Van Slyke's method, according to the technique of Plimmer and Rosedale [1925], except in the cases of pig uterus and placenta when the original method was followed, although in these analyses also, the determinations of amide- and humin-nitrogen were carried out in accordance with the procedure of Plimmer and Rosedale, and the estimation of arginine-nitrogen followed the modification of Plimmer [1916] in every case.

The main difficulty in the Kossel procedure is that loss occurs at each precipitation. The problem has been therefore to ascertain whether any of the precipitates, expecially those involving the formation of barium sulphate can be dispensed with. Various alterations have been attempted during the course of the work and the procedure finally adopted is as follows.

After removal of the portions for analysis according to Van Slyke, the bulk is acidified to 5 % with HCl and precipitated with phosphotungstic acid. The amount of phosphotungstic acid used was 15 g. for each 0·5 g. of nitrogen, the volume of the bulk being doubled in accordance with the Van Slyke procedure. The precipitation is carried out in a large flask which is heated in a water-bath to redissolve the precipitate. After standing for at least 48 hours, the precipitate, which contains, in addition to the diamino-acids, ammonia and humin, is filtered on a Büchner funnel and washed five times with 50 cc. of dilute HCl. The precipitate is then placed in a basin for the decomposition of the phosphotungstates, which is carried out by means of baryta gradually added with stirring until the solution is faintly alkaline. The precipitate of barium phosphotungstate, to which the humin adheres, is rapidly filtered off by suction and the filtrate which now contains the diamino-acids and ammonia, is freed from the latter by concentrating *in vacuo*. Plimmer and Rosedale [1925] showed that, in concentrating the alkaline diamino-fraction at this stage, loss occurred, which was presumably due to decomposition of the amino-acids. The above procedure has however been employed, as any loss at this stage is not greater than that sustained by an earlier removal of the ammonia which would entail the formation of an additional precipitate and washing of a large bulk of barium sulphate. The aim is to carry through the operations of decomposing the phosphotungstates, filtering and concentrating as rapidly as possible. The excess of barium is now removed quantitatively by means of sulphuric acid and filtered off. The filtrate is then acidified to 1·5 % with sulphuric or acetic acid and histidine precipitated by adding a saturated solution of silver sulphate or acetate to the hot solution. After standing for 24 hours, the precipitate of the silver salt of histidine is filtered off and treated in accordance with the usual Kossel method.

A further quantity of silver salt is added to the filtrate which is made alkaline with baryta to secure precipitation of arginine. The filtrate from the silver-arginine compound is freed from silver by acidifying to 5 % with HCl and filtering. Lysine is precipitated from the filtrate by phosphotungstic acid and treated from this point in accordance with the Kossel method.

Results.

In order to compare the results of the Kossel and Van Slyke methods, the figures have all been reduced to the percentage of the total nitrogen which they represent. As already stated, during the Kossel procedure the amide- and humin-nitrogens were not estimated, so that these amounts have been secured only during the Van Slyke estimations. The average of the results obtained by Kossel's method are given in Table I.

Table I. *Results by the modified Kossel method.*

Percentages of total nitrogen.

	Pork muscle	Pig uterus	Human arm	Human foot	Breast carcinoma
Histidine-N	1·5	2·0	2·0	2·0	2·5
Arginine-N	7·0	13·0	5·0	7·0	7·0
Lysine-N	6·5	3·0	6·0	6·0	3·0
No. of exps.	3	5	4	4	3

While the above figures have been presented to the nearest 0·5 %, divergencies have occurred in some of the determinations composing the average. The most noteworthy concern arginine, where the figures varied in the "foot" experiments from 6·1 to 8·2 %, and in the "arm" experiments from 4 to 6·1 %. The most notable figure in the above table is that of lysine in carcinoma, but in this case the figures for the three determinations, 3·3, 3·8, 3·0 % show no very marked divergence from one another. As will be seen from Table II, the Van Slyke estimation gives a very low figure.

Table II. *Results by Van Slyke's method.*

Percentages of total nitrogen.

	Amide-nitrogen	Humin-nitrogen	Diamino-N Total	Amino-nitrogen	Arginine-nitrogen	Histidine-nitrogen	Lysine-nitrogen	Mono-amino-nitrogen total	Total
Pig muscle	8·0	6·8	18·4	11·1	8·1	1·9	8·4	65·7	98·9
Pig uterus	8·5	8·7	18·0	9·6	8·3	3·2	6·5	64·2	99·4
Pig placenta	8·2	3·2	24·6	9·0	11·3	9·4	3·9	59·8	95·8
Pig foetus	7·5	14·8	15·4	8·8	8·5	0·3	6·6	58·0	95·7
Human arm	4·2	5·2	19·0	10·9	8·7	2·5	7·8	67·8	96·2
Human foot	4·4	5·0	18·3	9·9	9·0	2·5	6·8	68·1	95·8
Breast carcinoma ...	7·0	14·5	14·6	7·9	9·1	3·0	2·5	64·7	100·8
Chicken feathers ...	8·5	8·7	18·0	9·6	8·3	3·2	6·5	64·2	99·6
Maize-fed chicken flesh	8·5	8·7	13·4	7·6	8·7	2·6	2·1	66·8	97·4

In general, it will be seen that the Van Slyke method gives slightly higher results than the Kossel method. At the same time the difference if calculated in terms of the actual amino-acid is negligible. The conclusion may be drawn that normal muscle tissue does not vary to any extent in the various animals

or in different tissues of the same animal, provided the animal secures normal protein in the diet. The maize-fed chickens were taken from a group of experimental birds, which had received no protein other than that contained in maize. While maize contains other proteins than the deficient zein, it would appear that these are not present in sufficient quantity to produce normal flesh.

In Table III the amounts of the three diamino-acids in 100 g. of flesh are given.

Table III. *Diamino-acids, g. per* 100 *g. tissue.*

	Arginine	Histidine	Lysine
Pig muscle	1	0·27	1·7
Pig uterus	1	0·5	1·3
Pig placenta	1·2	1·1	0·9
Pig foetus	0·6	0·26	0·8
Human arm	0·75	0·25	0·8
Human foot	1	0·27	1
Breast carcinoma	0·63	0·24	0·3
Chicken feathers	3·71	1·7	4·9
Maize-fed chicken flesh	0·75	0·26	0·3

SUMMARY.

1. The diamino-acid content of several animal tissues has been determined by Van Slyke's method and by a modification of Kossel's method.

2. No fundamental differences occur in the two methods used.

3. It appears that the diamino-acid contents of the tissues of different animals, and of different parts of a normal animal are similar.

4. Low content of lysine has been found in carcinoma, and in chickens which have derived the whole of their food-protein from maize.

In conclusion, I desire to thank Prof. R. H. A. Plimmer most heartily for his guidance and advice throughout the course of this work, and also the Dean of this School and Dr M. J. Rowlands for the supply of material used.

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CV. STUDIES ON COLOUR TESTS FOR STEROLS AND VITAMIN A.

I. STEROL TESTS.

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Numerous colour tests for sterols have been put forward during the last fifty years, the reagents employed including concentrated sulphuric acid [Salkowski, 1872], concentrated sulphuric acid with acetic anhydride [Liebermann, 1885], acetyl chloride with zinc chloride [Tschugaieff, 1900], arsenic and antimony trichlorides [Kahlenberg, 1922], and antimony pentachloride [Steinle and Kahlenberg, 1926]. In some cases the production of colour is activated by addition of benzoyl peroxide [Lifschutz, 1898], nitric acid [Whitby, 1923] or formaldehyde [Whitby, 1923]. The initial colours obtained are either blue or red, or a blend of these.

The first colour test for vitamin A, put forward by Drummond and Watson [1922], was based on the old Salkowski [1872] reaction which had long been used to estimate the "activity" of cod-liver oil. Many years before vitamins had been discovered, this characteristic reaction with concentrated sulphuric acid had been shown to be due to a constituent residing in the unsaponifiable fraction of the oil, and destroyed by oxidation when the oil became rancid. Other "vitamin" reagents (*e.g.* arsenic trichloride [Rosenheim and Drummond, 1925], antimony trichloride [Carr and Price, 1926]) would also appear to be closely related to sterol tests, and give similar, though more transient, colours.

In view of these facts, and of the gradually accumulating evidence in favour of vitamin A being a sterol derivative, it was thought of interest to make a comparative study of some of these colour tests put forward for sterols and for vitamin A.

Experimental.

Sources of sterols employed.

The cholesterol derivatives were prepared by Mr W. A. B. Sexton, working at Liverpool University, from pure cholesterol obtained from cod-liver oil. Feeding tests showed this cholesterol to be free from vitamins A and D. The purity of these derivatives, and the absence of ergosterol, was spectroscopically controlled. The ergosterol was obtained from ergot, and the sitosterol from wheat germ oil. Analytical data obtained on these sterols and derivatives are given in Table I.

Reagents.

These were usually prepared and applied according to the directions of their respective authors. In certain cases modifications were introduced on account of factors such as time, temperature, nature of solvent, concentration, presence of catalysts, which have been found to influence the results obtained.

Table I. *Sterols and sterol derivatives examined.*

Sterol or derivative	Formula	M. Pt.	No. of double bonds	$[\alpha]_D^{20°}$
Cholesterol[1]	$C_{27}H_{45}OH$	148·5°	1	− 39·2°
Cholesteryl acetate	$C_{27}H_{45}COOCH_3$	114·5°	1	—
Cholesteryl chloride	$C_{27}H_{45}Cl$	96°	1	—
α-Cholesterylene	$C_{27}H_{44}$	78–79°	2	−102·1°
Cholestene	$C_{27}H_{46}$	92°	1	− 53·05°
ψ-Cholestene	$C_{27}H_{46}$	80°	1	+ 60·13°
Cholesterylmethylxanthogenic ester	$C_{27}H_{45}OCSSCH_3$	127°	—	—
Cholestenone	$C_{27}H_{44}O$	80°	2	—
Dicholesteryl ether	$(C_{27}H_{45})_2O$	194–195°	2	—
Hydroxycholesterylene	$C_{27}H_{42}O$	110°	3	—
β-Hydroxycholestenol acetate	$C_{27}H_{43}O_2COCH_3$	156°	—	—
Ergosterol	$C_{27}H_{42}O$	162°	3	−127°
Sitosterol	$C_{27}H_{46}O$	132°	—	− 34·4°

[1] Freed from ergosterol by boiling in alcoholic solution with norite for several hours, and fractional crystallisation.

Results.

These may be summarised as follows.

1. Pure cholesterol, freed from ergosterol, gives with the "vitamin" reagents (concentrated sulphuric acid, arsenic and antimony trichlorides) red colours persisting for many hours. Similar results are obtained with cholesteryl acetate and chloride, α-cholesterylene, cholestene and ψ-cholestene, but with the last two more time may be required for the colour to develop.

2. Cholesterol, cholesteryl acetate or chloride, cholestene and ψ-cholestene in chloroform solution, left in contact with concentrated sulphuric acid for some hours, and then diluted with more chloroform, give a purple or violet colour. Similar colours can be obtained by removal of the chloroform solution from the acid after less than a minute's contact, and addition to the former of a drop of formalin.

3. No colours are produced by formaldehyde alone with chloroform solutions of cholesterol or its derivatives which have not been in contact with concentrated sulphuric acid.

4. Irradiation of sterol derivatives generally has the effect of rendering the colours more transient. In the case of cholesterol, however, irradiation under certain conditions may develop the property of producing with the "vitamin" reagents blue colours, changing to red on standing for some hours.

5. Activation with other agents, such as acetic anhydride, benzoyl peroxide or formaldehyde, may lead to blue or purple colours being obtained on addition of the "vitamin" reagents.

6. Cholestenone, which has been suggested [Heilbron, Morton and Sexton, 1928] as being a typical product of irradiation, gives transient red colours with the "vitamin" reagents, and negative results (including red instead of blue with Liebermann's and Lifschutz's tests) with all other tests.

7. The other oxidation products of cholesterol which have been tested give negative results in all cases (dicholesteryl ether can be made to give red colours with modified Lifschutz's, Liebermann's, Tschugaieff's and "vitamin" tests).

8. Antimony pentachloride gives the colour sequence red → blue → red with all cholesterol derivatives examined except the oxidation products. Of these dicholesteryl ether, containing one proportion of oxygen, gives both red and blue, cholestenone and hydroxycholesterylene, containing two proportions of oxygen, give red only, and β-hydroxycholestenol acetate, containing four proportions of oxygen, gives negative results.

9. Introduction of sulphur into the side chain, as in cholesterylmethylxanthogenic ester, retards the development of the colours, but does not necessarily prevent it after liberation of sulphur compounds has taken place.

10. Ergosterol differs from cholesterol only in its results with the "vitamin" reagents. These give with ordinary concentrations of ergosterol the usual red, but if a higher initial concentration be employed (about 0·05 g. to 1 cc. of reagent) the red colour given by arsenic or antimony trichloride changes to purple or blue, on diluting with more reagent after a few moments.

11. Sitosterol gives similar results to cholesterol, but more slowly.

Details of the individual tests are given in Table II.

Table II. *Colour tests on sterols and derivatives.*

	"Vitamin" reagents									
Sterol or derivative	Salkowski, Drummond and Watson	Rosenheim and Drummond	Carr and Price	Liebermann	Lifschutz[1]	Rosenheim (methylal and $AsCl_3$)	Whitby *A*	Whitby *B*	Tschugaieff	Steinle and Kahlenberg
Cholesterol[2]	R	R	R	R → B	B → R	B → R	B	R[3]	R	R → B
Cholesteryl acetate	R	R	R	R → B	B → R	B → R	B	R	R	R → B
Cholesteryl chloride	R	R	R	R → B	B → R	B → R	B	R	R	R → B
α-Cholesterylene	R	R	R	R → B	P → R	B	B	R	R	R → P
Cholesterol, irradiated[4]	B → R*	B → R*	B → R*	R → B*	B → R*	B*	P	R	R	R → B
α-Cholesterylene, irradiated[5]	R	R	R	R → B	B → R	B	P	R	R	R → P
Cholestene	→ R	→ R	→ R	R → P	R	B	B	R	R	R → P
ψ-Cholestene	→ R	→ R	→ R	R → P	R	B	B	R	R	R → P
Cholesterylmethylxanthogenic ester	→ R	→ R	→ R	R → P	→ R	→ R	P	→ R	R	R → P
Cholestenone	→ R	→ R	→ R	R	R*	—	—	—	—	R
Dicholesteryl ether	→ R	→ R	→ R	→ R	R*	—	—	—	R*	R → B
Hydroxycholesterylene	—	—	—	—	—	—	—	—	—	R
β-Hydroxycholestenol acetate	—	—	—	—	—	—	—	—	—	—
Ergosterol	R	R[3] B → R	R[3] B → R	R → B	B	B	B	R	R	R → B
Sitosterol	R	R	R	R → B	B	B	P	R	R	R → B

[1] Heated in chloroform solution for a few hours at 37°, then cooled, and arsenic or antimony trichloride added.
[2] Freed from ergosterol as previously described.
[3] In high concentration the initial red colour changes rapidly to a blue which may persist for several hours.
[4] Irradiated half an hour at melting point.
[5] Irradiated until characteristic absorption bands disappeared.

"B" signifies a blue colour, "P" purple and "R" red.
→ Indicates development of colour on standing. * Denotes that the colour is more transient.

Discussion.

In the case of colour tests put forward for vitamin A, it was first suggested by Rosenheim and Drummond [1925], and Takahashi *et al.* [1925], that the initial blue colour obtained, rather than the red colour which develops on standing, is indicative of vitamin content. This initial blue colour has since been employed by a number of investigators to detect or estimate the vitamin in cod-liver oil and other sterol-containing natural products [Drummond, Channon and Coward, 1925; Carr and Price, 1926; Peacock, 1926; Rosenheim and Webster, 1926; 1927, 1, 2; Willimott, Moore and Wokes, 1926; Willimott and Wokes, 1927, 1, 2; Wokes and Willimott, 1927, 1, 2, 3]. Some interest, therefore, attaches to any case in which a sterol derivative produces with "vitamin" reagents blue colours similar to those attributed to the vitamin.

The results given above show that blue colours may be obtained from sterols under the following conditions:

(1) with antimony pentachloride;

(2) with acetic anhydride and concentrated sulphuric acid;

(3) with "vitamin" reagents on ergosterol in high initial concentration;

(4) with "vitamin" reagents on sterols treated with "oxidising" agents, formaldehyde or acetic anhydride.

It is true that these sterol colours are fairly stable, and may take a day or more to change to red or red-brown, whereas the "vitamin" colours are more transient, the blue colour in general having disappeared within a few minutes. But the above results show that irradiation of the sterols may cause the colours to be more transient, while it has been found possible, in the case of antimony trichloride on a physiologically tested cod-liver oil, to make the "vitamin" blue persist in measurable quantity for nearly an hour [Wokes and Willimott, 1927, 2].

Another characteristic property of the "vitamin" colours is their sequence, blue → red. In the case of antimony pentachloride, of acetic anhydride and concentrated sulphuric acid, and of arsenic or antimony trichloride on ergosterol in high concentration, the initial colour obtained is red. The blue → red sequence has, however, been obtained with cholesterol either irradiated at its melting point or oxidised under given conditions.

The case of ergosterol is exceptional in requiring a high initial concentration of sterol. Usually in sterol tests the most satisfactory concentration of sterol appears to be between 0·05 and 0·2 %. It has been shown by Wokes and Willimott [1927, 3] that the best results with antimony trichloride as a quantitative reagent for vitamin A in cod-liver oil are given by a concentration of oil of 1 to 5 %. Taking the unsaponifiable fraction of cod-liver oil as about 1 %, the optimum concentration of this fraction for "vitamin" tests would be from 0·01 to 0·05 %, or not very different from the optimum concentration for sterol tests.

Thus, of the four classes of blue colours given by sterols and their derivatives, it is those which are obtained by addition of "vitamin" reagents to cholesterol or certain of its derivatives or to ergosterol, after "oxidation" with benzoyl peroxide or nascent formaldehyde, which resemble most closely the "vitamin" colours. In regard to cholesterol itself (not purified from ergosterol) it was shown by Lifschutz many years ago [1908] that it gave with concentrated sulphuric acid, after treatment with benzoyl peroxide, a definite blue colour. Marston [1924] found that "oxycholesterol" prepared according to Lifschutz's method also gave a blue colour with arsenic trichloride, a reagent which had previously been shown by Kahlenberg [1922] to give a red colour with untreated cholesterol. Robertson [1925] came to the conclusion that when cholesterol is oxidised by Lifschutz's method the composition of the product varies, and found it more satisfactory to aerate in aqueous colloidal suspension at 100° in presence of an acetone extract of brain tissue. Rosenheim [1927] pointed out that cholesterol or ergosterol, after treatment with either benzoyl peroxide or nascent formaldehyde (obtained from methylal) will give blue colours with arsenic or antimony trichloride. Moore and Willimott [1927] showed that cholesterol, after heating in air to about 150° for half to two hours will give ultramarine colours, persisting some hours, with antimony trichloride. The same colours were obtained from cholesterol which had been heated in colloidal aqueous suspension at 90° for an hour or more, the presence of brain tissue extract not being found essential.

Turning to cholesterol derivatives, the new results here recorded may, perhaps, permit certain deductions to be drawn. Whitby [1923] suggested that in a typical sterol colour test the first stage consisted in the production of a colourless hydrocarbon, such as cholesterylene, by the action on cholesterol of the condensing agent (*e.g.* concentrated sulphuric acid), and that this product then coupled with a second substance (*e.g.* formaldehyde) to give the coloured product. But the writer has failed to obtain any colours with cholesterylene or cholestene when treated with formaldehyde only (either as formalin solution or produced in the nascent condition by warming a methylal solution of the sterol with dilute acid). This would seem to be evidence against Whitby's theory. The only noticeable result of the loss of the hydroxyl group was a distinct retardation in the rate of development of the colour in the case of cholestene and ψ-cholestene, but not in the case of cholesterylene, which contains an additional double bond. Variation in the position of the double bond, or replacement of the hydroxyl group by an acid radicle (acetate or chloride) made no appreciable difference to the colours obtained.

The most interesting results have been obtained with "vitamin" reagents on cholesterol which has either been irradiated or treated with oxidising or other agents, such as benzoyl peroxide, formaldehyde and acetic anhydride. From the recent work of Heilbron, Morton and Sexton [1928] the inference

might be drawn that when cholesterol is irradiated, dehydrogenation takes place, with formation of oxidation products similar to cholestenone. Tests applied by the writer to the latter compound have shown it to give with the "vitamin" reagents rather unstable red colours only, in contradistinction to initial blue colours obtained from irradiated cholesterol. A failure to obtain blue colours was also experienced after irradiation of cholesterylene and certain other cholesterol derivatives. These results do not necessarily imply that cholestenone is not a product of the irradiation of cholesterol, but there seems to be a strong possibility that, under suitable conditions, there is formed some other chromogen which is responsible for the initial blue colour. Further oxidation may lead to formation of chromogens giving red colours only, and finally to completely negative results. Oxidation of cholesterol can be made to give mixed products which when treated with "vitamin" reagents yield initial blue colours, changing gradually to red, which very closely resemble the "vitamin" colours given by the same reagents on cod-liver oil and other natural sources of vitamin A. But it has not yet been possible to isolate from these oxidation mixtures pure substances giving initial blue colours with the vitamin reagents, and only red chromogens have so far been obtained. With sterols as with the "vitamin" colours, the blue chromogen is unstable, and is destroyed by attempts to isolate it.

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CVI. THE EFFECT OF INANITION AND VITAMIN B DEFICIENCY ON THE ADRENAL GLANDS OF THE PIGEON.

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Introduction.

In a recent communication [Marrian *et al.*, 1927] it was shown that the hypertrophy of the adrenal glands which can be produced in pigeons by allowing them to feed voluntarily on a diet deficient in vitamin B, could also be produced by inanition even when liberal supplies of extracts rich in the vitamin were fed. It was at first thought that this indicated that the adrenal hypertrophy of vitamin B deficiency was entirely due to the state of inanition that almost invariably accompanies this condition. However, they found that when the pigeons were forcibly fed with the deficient diet, so that the loss of body weight at the conclusion of the experiment was negligible, the adrenals were enlarged to approximately the same extent.

The present work was undertaken in order to confirm and if possible find some explanation for these curious results. In addition it was thought desirable to investigate the possibility of a deficiency of one only of the two recently recognised factors in the vitamin B complex being involved in the causation of this hypertrophy.

Experimental.

The observations reported here were carried out on the same series of pigeons upon which a study of the effect of vitamin B deficiency and inanition on the testis was carried out [Marrian and Parkes, 1928].

Male birds were exclusively used, since the size of the adrenal glands seems to vary appreciably with sex, and in the case of the hen bird there would appear to be a definite hypertrophy during ovulation [Riddle, 1922].

The birds were then divided into five groups which were arranged and treated in the following manner:

Group	Diet	Vitamin preparations given	Object of experiment
A	20 g. vitamin B-deficient diet daily forcibly fed	1 g. yeast extract daily	Normal controls
B	" "	—	Vitamin B deficiency
C	" "	1 g. autoclaved yeast extract daily	Vitamin B_1 deficiency
H	" "	Peters's torulin curative dose × 3	Vitamin B_2 deficiency
SM	Starving	1 g. yeast extract daily	Inanition

The deficient diet used was an artificial one, of the type used by Randoin and Simmonet [1924]. The method of its preparation and the technique of forcible feeding have been described fully elsewhere [Kon and Drummond, 1927; Marrian *et al.*, 1927].

The vitamin preparations were administered to the birds in aqueous solution directly into the crop through a rubber catheter tube. The autoclaved yeast extract (known to contain no vitamin B_1) was prepared by heating a slightly alkaline solution of commercial yeast extract to 110° for 3 hours. Before use this was carefully neutralised. The preparation containing vitamin B_1 only was prepared according to the method of Kinnersley and Peters [1927].

In order to avoid errors in body weight due to overloaded crops, the birds were all starved for 24 hours before the initial weighings were made. At the conclusion of the experiments any food remaining in the crop was quantitatively removed, dried and weighed. The final weight of each bird was obtained by subtracting this figure from the total body weight immediately before death.

The vitamin B_1-deficient pigeons (groups *B* and *C*) were killed at the first signs of typical polyneuritis, while those in groups *A* and *H*, which would not be expected to show terminal symptoms were killed as soon after as possible. The starving pigeons were killed at the beginning of the final collapse, which has been described by Marrian *et al.* [1927].

Immediately after the death of the bird the adrenal glands were cut out, carefully dissected from any adhering connective tissue and fat, and weighed. Adrenaline was estimated in some of these by the modified Folin, Cannon and Denis method suggested by Baker and Marrian [1927]. Estimations of the moisture content of a number of others were carried out to investigate the possibility of the observed hypertrophy being to some extent due to oedema.

General behaviour of the birds.

A brief description only of the general behaviour of this series of pigeons will be made, since this has been dealt with elsewhere [Marrian and Parkes, 1928].

With one exception, which will be referred to in a later section of the paper, these birds behaved in a normal manner and in all cases the body weights were satisfactorily maintained. The majority of the birds in groups *B* and *C* showed the classical symptoms of neck retraction, but in a few instances the condition called emprosthotonus by McCarrison [1919] was observed. The group *H* birds, like those in groups *B* and *C*, vomited daily to some extent, but otherwise no definite symptoms could be observed.

Since the body weights of nearly all the birds in these groups were satisfactorily maintained, it is evident that the greater part of the diet was retained and satisfactorily metabolised.

The general condition of the pigeon during inanition has been fully discussed previously [Marrian *et al.*, 1927].

Details of the weight changes, symptoms, and times on the diets of the birds are shown in Table I.

Weight of the adrenal glands.

On referring to Table I it will be seen that the adrenals of the pigeons that were forcibly fed on the deficient diet and also those of the starving birds receiving vitamin B are definitely hypertrophied when compared with those of the forcibly fed controls in group *A*. This confirms the findings of Marrian *et al.* [1927].

The results from the birds in groups *C* and *H*, deficient in vitamins B_1 and B_2 respectively, are however not quite so clear cut. The average figure for the weight of the glands of the group *C* birds seems to indicate that the hypertrophy is of almost the same order as in group *B*. The weight of the adrenals of *C* 66 however was more than twice the average of the remainder in the group, and since this bird was abnormally oedematous it would almost seem justifiable to eliminate this result. If this is done, an average figure for the adrenal weight in group *C* of 0·0425 g. is obtained, which, although quite definitely greater than the control, is considerably less than the group *B* average.

The average weight of the adrenals of the group *H* pigeons is quite clearly significantly less than the group *B* average, but still definitely above that of the control group. The possibility must be borne in mind that these birds were vomiting an appreciable quantity of the antineuritic vitamin and that in consequence they were suffering from a mild vitamin B_1 deficiency. This however seems unlikely since the birds were receiving three times the normal curative dose daily by mouth and in addition were injected with the extract on three occasions.

The author is inclined to the view that this slight adrenal hypertrophy of the pigeons deficient in the thermostable factor is quite definite and also that the difference in degree of hypertrophy between groups *B* and *C* is real and significant. Thus it would appear that a deficiency of each of the two factors plays a part in the production of the adrenal enlargement observed in vitamin B deficiency, the vitamin B_1 deficiency being the more important.

This conclusion is in agreement with the recent observations of Findlay [1928] who has shown that the adrenal glands of rats are enlarged as a result of vitamin B_1 or B_2 deficiency. The greater degree of hypertrophy of the adrenals of Findlay's rats deficient in vitamin B_2 only, when compared with the group *H* pigeons, may be explained by the fact that they were fed on the deficient diet for over twice as long as those deficient in vitamin B_1, whereas the pigeons in groups *C* and *H* were receiving the deficient diets for approximately the same length of time.

Another possible explanation of these results is suggested by the recent

work of Williams and Waterman [1927] who on rather scanty evidence suggest that a third factor (present in whole wheat) is necessary for the satisfactory health and growth of the pigeon, vitamin B_2 being relatively unimportant. Assuming that it is present in yeast extract, it is conceivable that the adrenal hypertrophy might in some degree be due to a deficiency in this factor.

Table I.

Group	No.	Initial weight (g.)	Final weight (g.)	Weight change %	Days on diet	Weight adrenals (g.)	Total adrenaline (mg.)	Adrenaline per 1 g. gland (mg.)	Moisture content of adrenals %	Remarks on condition of bird when killed	
A	5	405	475	+19·7	18	0·0232	0·057	2·46	—	—	—
A	3	389	476	+22·4	25	0·0270	0·068	2·52	—	—	—
A	6	292	348	+19·1	26	0·0277	0·062	2·24	—	—	—
A	39	446	500	+12·1	22	0·0174	—	—	70·7	—	—
A	43	406	452	+11·3	22	0·0287	0·060	2·09	—	—	—
A	42	471	475	+ 0·8	23	0·0352	—	—	71·3	—	—
A	40	434	443	+ 2·1	24	0·0294	—	—	74·7	—	—
A	38	421	440	+ 4·3	25	0·0268	—	—	72·0	—	—
A	44	346	420	+17·6	25	0·0248	—	—	70·5	—	—
A	37	411	390	− 5·1	26	0·0297	—	—	73·3	—	—
A	Average	402	442	+10·4	24	0·0270	0·062	2·33	72·1		
B	7	394	398	+ 1·0	17	0·0539	0·066	1·22	—	Severe neck retraction	
B	9	382	352	− 7·8	18	0·0813	0·172	2·12	—	Neck retraction	
B	8	401	434	*	18	0·0696	0·078	1·12	—	Neck retraction. Breast very oedematous	
B	11	350	354	+ 1·1	21	0·0810	0·138	1·17	—	Emprosthotonus	
B	10	343	279	−18·6	22	0·0515	0·063	1·22	—	Neck retraction	
B	52	423	411	− 2·8	16	0·0586	—	—	75·2	Weak. Slight neck retraction	
B	45	406	393	− 3·2	17	0·0573	—	—	76·1	Severe neck retraction	
B	51	516	456	−11·6	19	0·1062	—	—	75·9	Neck retraction	
B	50	431	355	−17·6	19	0·1023	—	—	75·6	Emprosthotonus	
B	48	424	350	−17·4	24	0·0424	—	—	75·5	Severe neck retraction	
B	Average	407	378	− 7·7	19	0·0704	0·103	1·37	75·7		
C	18	394	386	− 2·0	22	0·0376	0·051	1·36	—	Neck retraction	
C	57	348	368	+ 5·7	18	0·0441	0·045	1·02	—	Emprosthotonus	
C	59	439	444	+ 1·0	22	0·0677	0·051	0·74	—	Emprosthotonus	
C	66	427	519	*	15	0·1222	0·122	1·00	—	Neck retraction. Breast extremely oedematous. Abdominal cavity full of fluid	
C	61	320	325	+ 1·6	16	0·0294	0·038	1·29	—	Emprosthotonus	
C	64	435	481	+10·5	18	0·0399	0·102	2·55	—	Severe neck retraction	
C	65	430	419	− 2·5	20	0·0360	0·062	1·72	—	Neck retraction	
C	Average	399	420	+ 2·4	19	0·0538†	0·067	1·38	—		
H	70	372	324	−12·8	23	0·0508	0·084	1·65	—	—	—
H	68	395	407	+ 2·9	23	0·0282	0·063	2·23	—	—	—
H	69	345	320	− 7·2	23	0·0330	0·073	2·21	—	—	—
H	72	441	374	−15·0	23	0·0345	0·105	3·03	—	—	—
H	67	371	336	− 9·4	23	0·0343	0·091	2·65	—	—	—
H	Average	385	352	− 8·3	23	0·0361	0·083	2·35	—	—	—
SM	75	312	204	−34·6	11	0·0358	0·088	2·46	—	—	—
SM	73	324	202	−37·7	17	0·0526	0·122	2·32	—	—	—
SM	74	387	237	−38·8	17	0·0533	—	—	79·2	—	—
SM	76	435	273	−37·2	21	0·0523	—	—	77·2	—	—
SM	78	478	285	−40·4	22	0·0528	0·137	2·59	—	—	—
SM	Average	387	240	−37·7	18	0·0494	0·116	2·46	78·2		

* Both these birds were extremely oedematous, hence the body weight changes are of little significance.
† 0·0425, excluding pigeon *C* 66.

Oedema of the adrenal glands.

A number of determinations of the moisture content of the adrenals of the groups *A*, *B* and *SM* birds were carried out in order to see to what extent the adrenal enlargement in the two latter groups could be accounted for by an increase in the water content of the glands. Table I clearly shows that in both cases the glands were quite distinctly oedematous.

In the case of the starving birds this oedema was so marked as to account for nearly half of the total observed hypertrophy.

The following table summarises these results.

Table II.

Group	Fresh weight adrenals g.	Dry weight adrenals g.	Water %	Hypertrophy calculated on fresh weight %	Hypertrophy calculated on dry weight %	Proportion of hypertrophy due to oedema %
A (av. of 6)	0·0272	0·0075	72·1	—	—	—
B (av. of 5)	0·0734	0·0178	75·7	169	137	19
SM (av. of 2)	0·0528	0·0115	78·2	94	53	44

The nature of the adrenal hypertrophy.

Several observers seem to be agreed that during inanition there is an increase in adrenaline roughly corresponding to the enlargement of the gland in the pigeon [McCarrison, 1919, 1920; Marrian *et al.*, 1927]. The three adrenaline estimations carried out on the *SM* group of pigeons seem to confirm these observations, although the experiments are admittedly too few in number. So far as the author is aware, no histological study has yet been made of the adrenal glands of starving pigeons, so that it is as yet impossible to say definitely whether this represents an actual proliferation of the medullary cells, a hyperactivity of the medulla which remains constant in size, or a decreased output of adrenaline. As a result of studies carried out on pigeons fed on polished rice, Kellaway [1921] explains the increased adrenaline content of the hypertrophied glands by postulating that the adrenaline output is decreased by reason of the lowered metabolism of the bird. This however seems to be unlikely since at the time when the enlarged gland shows a high adrenaline content, the cloacal temperature of the bird is not necessarily markedly subnormal. In the case of the adult rat Vincent and Hollenberg [1921] showed that the adrenaline content of the adrenals is increased after 2–3 days' starvation, and at this early stage of inanition the body temperature and metabolism of the rat are not greatly reduced [Drummond and Marrian, 1926]. McCarrison [1920] regards the increased adrenaline content of the glands as representing true medullary hypertrophy. This is regarded as being the more acceptable hypothesis.

A discussion of the nature of the hypertrophy resulting from vitamin B deficiency is rendered difficult owing to the facts that many of the birds

examined by previous workers have been virtually starving at the time of incidence of the nervous symptoms and that much of the evidence is apparently conflicting. Thus McCarrison [1919] examined starving pigeons that had lost an average of 23 % of their body weight, while his polished rice birds had lost about 30 %. It is not surprising therefore that these birds gave a similar series of results to his starving ones. Kellaway [1921] made a study of pigeons fed voluntarily and forcibly on polished rice. In both cases he showed that there was a marked increase in the adrenaline content of the glands. His forcibly fed birds, however, although showing a loss of body weight (15 %) were far from being in a state of inanition. Beznak [1923] on the other hand, could find no increased amount of adrenaline in the hypertrophied glands of polyneuritic pigeons that had lost as much as 22 % of their original body weight.

The histological evidence suggests that it is the cortex that is mainly involved in the hypertrophy [Findlay, 1921; Lasowsky and Sumnitzki, 1926]. Findlay indeed showed that after curing polyneuritic pigeons with yeast, there occurred a rapid decrease in the cortical lipoids. It seems fairly clear therefore that in experimental beriberi changes do occur in the adrenal cortex.

Marrian *et al.* [1927] showed that in five forcibly fed vitamin B-deficient pigeons, whose average loss of body weight was only 5 %, the adrenals were considerably hypertrophied while the adrenaline content was not increased. It was pointed out that this might be explained on the assumption that the cortex alone hypertrophied when starvation did not complicate the picture.

However in the five group *B* pigeons that were examined, two cases (*B* 9, *B* 11) were found where the adrenals contained an abnormally large amount of adrenaline. The other three cases were entirely in line with the results of Marrian, Baker, Drummond and Woollard. It seems to be necessary therefore to conclude that in certain cases of experimental beriberi where the adrenaline content of the glands is increased, even when the starvation factor is eliminated, the medulla may be involved in the hypertrophy to some extent.

In spite of the somewhat disturbing nature of these results the bulk of the evidence certainly seems to indicate that the cortex is the site of the adrenal hypertrophy in most cases of vitamin B deficiency, the medulla being involved when starvation complicates the picture and also in certain other random cases.

This hypothesis receives some support from recent work on the function of the adrenal cortex. Evidence based on observations on adrenalectomised animals is accumulating to show that the adrenal cortex has the function of removing or neutralising in some manner various toxins, bacterial or otherwise, in the body [Lewis, 1923; Jaffe, 1926; Banting and Gairns, 1926; Belding and Wyman, 1926; Torino and Lewis, 1927], although this theory is strenuously denied by Stewart, Rogoff and their collaborators in a series of papers.

It also seems to be an established fact that bacterial infections and certain types of intoxication may cause an enlargement of the adrenal cortex.

It is interesting to note that McCarrison [1919] showed that in polyneuritic pigeons that were also suffering from various bacterial infections the adrenals were considerably enlarged without any corresponding increase in the amount of adrenaline. Since the symptoms in such birds were observed at an earlier date than in uninfected ones, one might expect that the starvation factor would be decreased or altogether eliminated. Thus the hypertrophy might be due either to vitamin B deficiency, or to the bacterial toxin, or more probably to both.

Origin of the nervous symptoms arising from vitamin B deficiency.

The view has often been expressed that the underlying cause of the nervous symptoms observed in vitamin B deficiency is a toxic substance. Thus one might build up an attractive theory explaining the adrenal hypertrophy in beriberi as an attempt by the body to eliminate the "beriberi" toxin. It should be possible to test this theory by showing that adrenalectomised animals develop beriberi more rapidly than do normal animals. Several unsuccessful attempts have been made by the author to do this. A few initial experiments were carried out on rats and in one instance convulsive seizures were observed a few days after the animal had been placed on the deficient diet. There were no grounds to believe however that these convulsions were specifically due to the vitamin deficiency, since convulsions are often a symptom of adrenal insufficiency in normally fed animals. In addition it has been shown by the author that adrenalectomised rats survive starvation for a much smaller period than normal rats (unpublished work), and since marked loss of appetite is a symptom both of adrenal insufficiency and vitamin B deficiency, it seemed to be useless to continue such experiments.

Beriberi-like symptoms in normal pigeons.

An extraordinary case was observed among the group *A* pigeons, which were receiving 1 g. of yeast extract daily. On the 19th and 20th days it was noticed that pigeon *A* 4 was far from well. After each meal there was considerable vomiting and in general it may be said that the condition of the bird closely resembled that of the vitamin B-deficient pigeons just before the incidence of the nervous symptoms. On the morning of the 21st day this bird was found in a condition absolutely indistinguishable from the usual symptoms of experimental "beriberi." The head of the bird was severely and persistently retracted and there was a periodic tendency to turn "cartwheels." It would be no exaggeration to say that this bird seemed to be a more characteristic case of "beriberi" than many of those fed on deficient diets. The initial weight of the pigeon was 389 g. At the time that the symptoms appeared the total body weight was 433 g. The weight of food in

the crop was judged to be no more than 30 g., so evidently no loss of body weight had occurred.

It appeared extremely unlikely that this curious condition could be caused by a vitamin deficiency resulting from the vomiting of the administered yeast extract since no vomiting had been noticed until the 19th day. Even if the whole of the yeast extract had been expelled on the 19th and 20th days, it is quite inconceivable that this short period of deficiency could be the cause of the symptoms.

After some hours, as the condition remained unchanged, it was decided to see if the condition was curable by an injection of antineuritic vitamin. Accordingly 1 cc. of an extract made by the method of Kinnersley and Peters, that was known to be curative in doses of 0·5 cc., was injected. After 3 hours the pigeon appeared to be no better. The following morning the symptoms were still severe although the bird appeared considerably weaker. It was quite evident therefore that this condition was not curable by vitamin B_1. The bird was subsequently killed while still showing these symptoms.

This case goes to strengthen the belief, which is rapidly growing among workers on beriberi in the pigeon, that the symptom of neck retraction is not specific to vitamin B deficiency and in fact may occur as a result of a variety of causes. Thus Marrian *et al.* [1927] observed symptoms of neck retraction in starving pigeons that were receiving ample supplies of yeast extract, and Williams [1927] reports a similar condition in pigeons that were fed exclusively on rice polishings. The author has also observed on one occasion well-defined neck retraction in a normal pigeon that was partially anaesthetised with ether.

Summary.

1. The results obtained by Marrian, Baker, Drummond and Woollard showing that adrenal hypertrophy occurred in starving pigeons receiving vitamin B and also in pigeons forcibly fed on an artificial vitamin B-free diet to exclude the starvation factor, have been confirmed.

2. The hypertrophy occurring in the vitamin B-deficient pigeons seems to be mainly due to a deficiency in vitamin B_1, but it is considered that a deficiency of vitamin B_2 is also a contributory factor.

3. The adrenal glands of both the starving and the vitamin B-deficient pigeons are oedematous. This oedema accounts for 19 % of the total hypertrophy shown by the vitamin B-deficient birds and 44 % in the case of the starving pigeons.

4. The question whether the adrenal hypertrophy occurring in starvation and vitamin B deficiency is medullary or cortical is discussed. It is considered possible that in starvation the changes occur mainly in the medulla while in vitamin B deficiency the cortex is mainly concerned. In certain cases changes in the medulla may occur in vitamin B deficiency.

5. A brief reference to a pigeon, forcibly fed on a complete diet, that developed acute symptoms resembling those due to vitamin B deficiency is made. This condition, although identical in appearance with avian beriberi, was not curable by vitamin B_1.

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CVII. DETERMINATION OF HYDROGEN ION CONCENTRATIONS IN PHOSPHATE AND BORATE MIXTURES BY MEANS OF THE QUINHYDRONE ELECTRODES.

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I. Introduction.

The application of the quinhydrone electrode (or the quinoquinhydrone electrode or the hydroquinhydrone electrode) as substitute for the hydrogen electrode for the determination of p_H is based on the fact that the values of the potentials between one of these electrodes and the hydrogen electrode vary but very little if the electrolytes of the two electrodes of the chain examined are identical. For aqueous electrolytes at 18° the following values as a rule may be used:

quinhydrone-hydrogen	0·7042 volt
quinoquinhydrone-hydrogen	0·7562 ,,
hydroquinhydrone-hydrogen	0·6176 ,,

The potential shown above of the simple quinhydrone electrode being only valid for dilute solutions (up to 0·1–0·2 *M*), the constants of the quinoquinhydrone and hydroquinhydrone electrodes should also be valid at higher concentrations, and this has been verified by means of experiments [Biilmann, 1921, 1927; Biilmann and Lund, 1923].

As these electrodes are often applied in biological researches, it seems important to know how they act in phosphate and borate mixtures, and researches on these solvents have only been made with a simple quinhydrone electrode.

In the following we shall first give the determinations of the fundamental potentials which may be used for the application of the different quinhydrone electrodes in phosphate and borate solutions. The solutions examined are:

Phosphate mixtures.

1/15 *M* KH_2PO_4 cc.	1/15 *M* Na_2HPO_4 cc.	p_H
900	100	5·90
700	300	6·45
500	500	6·81
300	700	7·16
200	800	7·38
100	900	7·73
50	950	8·04

Borate mixtures.

1/5 *M* H_3BO_3 1/20 *M* NaCl cc.	1/20 *M* $Na_2B_4O_7$ cc.	p_H
900	100	7·34
800	200	7·76
700	300	8·05

Further, we have examined some phosphate and bicarbonate solutions saturated with carbon dioxide, and finally we shall investigate the influence of these new determinations on the results of the researches which Biilmann and Katagiri [1927] and Grossman [1927] have recently published in this *Journal*. Before dealing with the experiments, it must be remembered that in the simple quinhydrone electrode the solution examined is saturated with quinhydrone, in the quinoquinhydrone electrode with quinone and quinhydrone and in the hydroquinhydrone electrode with quinol and quinhydrone. Whereas quinhydrone is not very soluble in water (about 0·02 *M*) and the saturated solution of quinone in water is only about 0·1 *M*, the saturated solution of quinol is about 0·5 *M* at 18° [Biilmann, 1927; Biilmann and Lund, 1923].

II. The quinhydrone electrode.

The potential of the quinhydrone electrode compared with the hydrogen electrode in the same electrolyte is generally assumed to be 0·7042 volt at 18°. This value gives very good determinations of p_H in many dilute solutions of acids or mixtures of acids and salts. Now we have examined the quinhydrone electrode in the phosphate mixtures mentioned above and found the values recorded in Table I.

Table I.

+ Quinhydrone, phosph. mixt. | phosph. mixt., H_2 −. 18°.

p_H	5·90	6·45	6·81	7·16	7·38	7·73	8·04
π	0·7060	0·7057	0·7056	0·7058	0·7058	(0·7062)	(0·7072)

Mean value: 0·7058.

At p_H 7·73 and 8·04 the potentials are not so stable as in the more acid solutions. The measurements show that when p_H in phosphate mixtures is determined with the quinhydrone electrode, the value 0·7058 ought to be used for the calculation instead of 0·7042 which will give p_H values about 0·03 too low. When measuring the borate mixtures at 18°, the values given in Table II were obtained.

Table II.

+ Quinhydrone, borate mixt. | borate mixt., H_2 −. 18°.

p_H	7·34	7·76	8·05
π	0·7049	0·7041	0·7046

Mean value: 0·7045.

The mean value 0·7045 agrees so well with 0·7042 that the difference only amounts to 0·005 in the p_H value.

III. The quinoquinhydrone electrode.

It is to be remembered that the quinoquinhydrone electrode is saturated both with quinone and with quinhydrone. In phosphate mixtures at 18° the values recorded in Table III were found.

Table III.

+ Quinone, quinhydrone, phosph. mixt. | phosph. mixt. H_2 −. 18°.							
p_H	5·90	6·45	6·81	7·16	7·38	7·73	8·04
π	0·7562	0·7560	0·7561	0·7562	(0·7565)	(0·7565)	(0·7530)

At p_H 7·38, 7·73 and 8·04 the potentials drifted so rapidly that exact determinations could not be obtained, and on the whole the determinations with these electrodes had to be made soon after mounting the electrodes. The mean of the first four potentials, 0·7561 volt, agrees well with the values found by Biilmann and Lund [1923] in hydrochloric acid and sulphuric acid (0·7561–0·7564, mean 0·7562) and with the value 0·7564 found by Schreiner [1925] in 2 *N* sulphuric acid.

In borate mixtures p_H 7·34–8·05 the potentials drifted so rapidly that exact readings could not be taken.

IV. The hydroquinhydrone electrode.

In the hydroquinhydrone electrode the solution is saturated both with quinol (hydroquinone) and with quinhydrone. The potentials compared with those of the hydrogen electrode in phosphate mixtures are shown in Table IV.

Table IV.

+ Quinol, quinhydrone, phosph. mixt. | phosph. mixt., H_2 −. 18°.							
p_H	5·90	6·45	6·81	7·16	7·38	7·73	8·04
π	0·6229	0·6221	0·6229	0·6238	0·6253	0·6304	0·6411

From the table we see that π is not constant, but increases considerably when the p_H of the solution examined increases from 5·90 to 8·04. This is not due to an influence of the phosphate mixtures on the activities of quinone and quinol, as these are constant in the saturated solutions. Accordingly, it is to be assumed that the quinol exercises an influence on the phosphate mixtures changing their p_H values increasingly with increasing p_H values, which gives rise to corresponding changes of the potentials. An influence of this kind may *a priori* be assumed for the basic solutions, as quinol is a weak acid, but it will be seen that the acid effect of the quinol does not alone account for the observed changes in p_H. An influence of quinol on the activity of the ions may, therefore, also take place.

In borate mixtures we found an influence similar to that in phosphate mixtures (Table V), but later we shall see that in this case we have only or chiefly to do with an acid effect of the quinol.

Table V.

+ Quinol, quinhydrone, borate mixt. | borate mixt., H_2 −. 18°.			
p_H	7·34	7·76	8·05
π	0·6264	0·6277	0·6296

In order to determine the influence of quinol on the p_H value of a phosphate mixture we first tried to measure the potential between an ordinary hydrogen electrode in the phosphate mixture and a hydrogen electrode in the same phosphate mixture saturated with quinol. But hydrogen electrodes turned out to give no constant and reproducible values in phosphate-quinol mixtures. Two electrodes mounted in the same electrode vessel differed by 5–6 millivolts, and besides the electrodes were considerably polarised during the readings. On the other hand, stable and reproducible potentials were obtained by means of the electrode recently described by Biilmann and Klit [1927]. In this electrode colloidal palladium catalyses the reversible transformation hydrogen-hydrogen ion, and the electrodes are not platinised but simply bright platinum foils. The different ways in which the two electrodes comport themselves may be explained by the different efficiencies of the two electrodes, as in the electrodes the feeble traces of quinone (or quinhydrone) always present in quinol have to be reduced to quinol down to a quinone concentration which is quite below analytical limits, before stable potentials will be obtained. In a hydrogen electrode containing quinol we have also a quinolquinone electrode, and these two electrodes will only produce the same potential, when, by the reduction of the traces of quinone, equilibrium corresponding to the following reversible reaction is obtained.

$$\text{Quinol} \rightleftharpoons \text{Quinone} + H_2.$$

At a hydrogen pressure of 1 atmosphere in the state of equilibrium the ratio conc. quinol/conc. quinone should be about 10^{24}.

The stable and well-defined potential of the palladium hydrogen electrode in the phosphate quinol mixture was about 0·03 volt more positive than the unstable potential of the ordinary hydrogen electrode in the same mixture.

Tables VI and VII show the potentials between hydroquinhydrone electrodes in phosphate and borate buffers and the palladium hydrogen electrodes in the same buffers saturated with quinol.

Table VI.

+Quinol, quinhydrone, phosph. mixt. | phosph. mixt. sat. quinol, Pd, H_2 –. 18°.

p_H of buff. mixt.	5·90	6·45	6·81	7·16	7·38	7·73	8·04
π	0·6194	0·6190	0·6189	0·6195	0·6193	0·6189	0·6194

Mean value: 0·6192.

Table VII.

+Quinol, quinone, borate mixt. | borate mixt. sat. quinol, Pd, H_2 –. 18°.

p_H of borate mixt.	7·34	7·76	8·05
π	0·6213	0·6201	0·6193

The figures show that in borate mixtures no mean value of π can be obtained, while in the phosphate mixtures the values of π are found about a mean value 0·6192. For the potentials of hydroquinhydrone electrodes to

hydrogen electrodes in solutions of hydrochloric or sulphuric acid Biilmann and Lund [1923] have found values from 0·6176 to 0·6181.

The depression Δp_H of the p_H value of the buffers produced by the saturation of the buffer with quinol may be determined by measuring the potential $\Delta\pi$ between the palladium-hydrogen electrode in a buffer mixture saturated with quinol and the ordinary hydrogen electrode in the same buffer mixture *without* quinol. Table VIII shows the values of $\Delta\pi$ and Δp_H for the buffer mixtures with which we are dealing, the original p_H of these being recorded in the first column of the table. In order to show variations, the p_H values (determined carefully with hydrogen electrodes) are given to 3 decimals; further, the total quinol concentration is recorded, the quinol being determined by titration with iodine in bicarbonate alkaline solution.

According to the definition of p_H the true p_H value of the buffer mixtures saturated with quinol is $(p_H - \Delta p_H)$. From the true p_H values, the quinol concentrations and the first dissociation constant of quinol it is possible to calculate with fair accuracy the influence which the quinol as an acid has on the p_H value of the mixtures. $\Delta' p_H$ in Table VIII gives the figures. The values of $\Delta'' p_H = \Delta p_H - \Delta' p_H$ show that influences other than that of the acidity of quinol affect the determinations in the phosphate mixtures and that the matter seems rather complicated, while in the borate mixtures $\Delta' p_H$ agrees fairly well with Δp_H.

Table VIII.

p_H	$\Delta\pi$	Δp_H	$\Delta' p_H$	$\Delta'' p_H$	Quinol, molarity
		Phosphate mixtures.			
5·896	0·0030	0·052	0·003	0·049	0·479
6·452	0·0035	0·061	0·006	0·055	0·472
6·811	0·0034	0·059	0·011	0·048	0·501
7·158	0·0040	0·069	0·031	0·038	0·526
7·382	0·0054	0·094	0·078	0·016	0·534
7·725	0·0108	0·187	0·177	0·010	0·540
8·040	0·0215	0·372	0·380	−0·007	0·555
		Borate mixtures.			
7·338	0·0053	0·092	0·095	−0·003	0·480
7·760	0·0077	0·133	0·132	0·001	0·493
8·054	0·0104	0·180	0·183	−0·003	0·507

The measurements show that the hydroquinhydrone electrode is not as a rule applicable for determinations of p_H in phosphate and borate mixtures. On the other hand, in certain intervals of p_H, it may be applied, when the right constants or suitable comparison electrodes are employed. So it may be possible to use the hydroquinhydrone electrode when measuring against another hydroquinhydrone electrode in a phosphate mixture not differing essentially from the liquid examined, a method made use of by Biilmann and Katagiri [1927].

V. Comparison of the different electrodes.

Table IX shows determinations of p_H in the same mixtures with quinhydrone, quinoquinhydrone and hydroquinhydrone electrodes as well as with ordinary hydrogen electrodes and with the colloidal palladium hydrogen electrodes. The electrodes were measured against the quinhydrone standard electrode with p_H 2·029 described by Veibel [1923].

According to the potentials given in sections II–IV the p_H values at 18° are calculated by means of the formula

$$p_H = 2{\cdot}029 + \frac{\pi + \pi'}{0{\cdot}05774},$$

in which π is the measured potential and π' has the following values according to the electrode type employed.

Hydrogen or coll. Pd, hydrogen electrode	$\pi' =$ −0·7042
Quinhydrone: in phosph. mixt.	$\pi' = 0{\cdot}7058 - 0{\cdot}7042 =$ 0·0016
in borate mixt.	$\pi' = 0{\cdot}7045 - 0{\cdot}7042 =$ 0·0003
Quinoquinhydrone	$\pi' = 0{\cdot}7561 - 0{\cdot}7042 =$ 0·0519
Hydroquinhydrone	$\pi' = 0{\cdot}6192 - 0{\cdot}7042 =$ −0·0850

The table shows the figures from two series of measurements I and II, quite independent of each other and made with two preparations of the different buffer mixtures.

Table IX. p_H *determined with different electrodes.*

Hydrogen electrode		Quinhydrone electrode		Quinoquinhydrone electrode		Hydroquinhydrone electrode		Coll. Pd, H_2 sat. quinol	
I	II	I	II	I	II	I	II	I	II
				Phosphate mixtures.					
5·90	—	5·89	—	5·90	—	5·83	—	5·85	—
6·45	6·46	6·46	6·47	6·46	—	6·40	6·41	6·39	6·41
6·81	6·81	6·81	6·81	6·81	6·82	6·75	6·76	6·75	6·76
7·16	7·17	7·16	7·17	7·16	7·18	7·08	7·09	7·09	7·09
7·38	7·39	7·39	7·39	7·38	(7·44)	7·28	7·30	7·29	7·31
7·73	7·73	7·72	7·73	—	—	7·53	7·55	7·54	7·53
8·04	8·04	8·02	8·01	—	—	7·66	7·69	7·67	7·67
				Borate mixtures.					
7·34	7·34	7·33	7·34	—	—	—	—	7·25	7·25
7·76	7·75	7·75	7·75	—	—	—	—	7·63	7·61
8·05	8·06	8·05	8·07	—	—	—	—	7·87	7·88

The agreement between the hydrogen electrode and the quinhydrone electrode as well as the quinoquinhydrone electrode is excellent in the phosphate mixtures examined, and the quinhydrone electrode gives exact values in the borate mixtures.

The p_H values found with the hydroquinhydrone electrode in phosphate mixtures are not to be compared with those found with the hydrogen electrode, but with the values found with the colloidal palladium hydrogen electrode, and it will be seen that the figures agree excellently. *Of course none of these electrodes gives the p_H as it was before the saturation with quinol, but the p_H of the mixture after saturation with quinol.*

As to the borate mixtures, no determinations of p_H could be made with quinoquinhydrone or hydroquinhydrone electrodes, because no mean values of the potentials between these electrodes and the hydrogen electrode (see section IV) and the colloidal palladium hydrogen electrode respectively (see Table V) could be determined.

VI. Remarks on Biilmann and Katagiri's paper [1927] and on F. Grossman's paper [1927].

Biilmann and Katagiri have applied the hydroquinhydrone electrode to some determinations of p_H in liquids containing glucose. They made use of a phosphate mixture, the p_H of which was found to be 6·79 by means of the quinhydrone electrode. According to the reduction potential of the quinhydrone in phosphate mixtures determined in section II, the true p_H value of the said solution is calculated to be 6·81, which agrees completely with the value determined with the hydrogen electrode (see Table IX).

In the use of the hydroquinhydrone electrode Biilmann and Katagiri have not introduced any correction for the acid effect of quinol, and this is quite allowable, as the p_H values of the mixtures are 6·81 or less, and, at 6·81, the correction would only amount to 0·01. On the other hand, the influence of quinol on the activities of the ions stated in the present paper has to be considered. In many of the measurements this influence is automatically eliminated, as Biilmann and Katagiri employed a hydroquinhydrone electrode containing an electrolyte of almost the same kind as the liquid examined. But as far as solutions saturated with CO_2 are concerned, the influence of quinol on such solutions cannot be judged from the measurements recorded above in the present paper.

In order to find out the p_H values of phosphate mixtures saturated with CO_2 we have calculated the value by means of the constants of carbonic and phosphoric acids as well as determined the p_H value with the ordinary quinhydrone electrode.

Table X.

p_H of Na_2HPO_4 solutions saturated with CO_2 at 735 mm. Hg. 18°.

Na_2HPO_4	0·025	0·050	0·100 *M*
Biilmann and Katagiri	6·05	6·26	6·45
Calculated	6·02	6·22	6·42
Quinhydrone	6·01	6·21	6·39
Biilmann and Katagiri corr.	6·01	6·22	6·41

Table X deals with the solutions treated in Table VIII of the paper by Biilmann and Katagiri. The values from the cited Table VIII are shown in the first row. The second row shows the calculated values and the third the values determined by means of the simple quinhydrone electrode. The figures show that the p_H values found by Biilmann and Katagiri are about 0·04 too high. The origin of this difference is that Biilmann and Katagiri, in their paper, assume the p_H of the phosphate hydroquinhydrone electrode to be 6·79 at 18°, but its true value is 6·75 as shown above. The hydroquinhydrone

having no, or only a slight, influence on the p_H of the phosphate mixture saturated with CO_2, the true p_H value of the comparison electrode ought to be used. The fourth row shows the p_H values from the measurements of Biilmann and Katagiri corrected in this manner.

Table XI.

p_H of $NaHCO_3$ solutions saturated with CO_2 at 740 mm. Hg. 25°.

$NaHCO_3$	0·025	0·050	0·100 *M*
Biilmann and Katagiri	6·22	6·45	6·74
Calculated	6·13	6·40	6·66
Quinhydrone	6·11	6·37	6·64
Biilmann and Katagiri corr.	6·15	6·38	6·67

Table XI deals, in the same manner, with Biilmann and Katagiri's experiments with bicarbonate solutions saturated with CO_2 at 25° (Table XII of the paper quoted). The table shows that the true p_H values in this case are about 0·07 lower than those found by Biilmann and Katagiri. This agrees with the fact that the p_H of the phosphate mixture of the comparison electrode saturated with quinol at 25° is not 6·79 but 6·72 as now found by means of the colloidal palladium electrode. The accordance between the figures in Table XI is not as good as in Table X, but suffices to show the order of magnitude of the correction which has to be introduced.

According to the facts mentioned in this section the electrometrically determined p_H values of solutions saturated with CO_2 in Biilmann and Katagiri's paper ought to be corrected. The true p_H values of the solutions saturated with CO_2 at 18° are 0·04, and those saturated with CO_2 at 25° are 0·07 lower than indicated, while the differences between the p_H values in each series of measurements are not affected by the correction.

Finally we shall make some remarks concerning the paper of Grossman [1927] on the use of the hydroquinhydrone electrode for p_H determinations in the fluids of the organism. Grossman has observed that the directly measured p_H values between 7 and 8 need a correction Δp_H, and for this correction he gives the formula

$$\Delta p_H = 0{\cdot}24 \times 2^{p_H - 7} - 0{\cdot}21,$$

where p_H in the exponent is the directly found p_H value. It seems to us that a formula of this type will only hold good for a certain buffer in a certain concentration, as the acid influence of the quinol may depend on the buffer effect of the examined mixture. A direct comparison with our measurements is difficult, as Grossman has carried out his experiments at different temperatures and the potentials of the hydroquinhydrone electrodes depend on the temperature. So we found for the chain

H_2 - phosphate mixture p_H 6·81 - hydroquinhydrone

the potential 0·6229 volt at 18°, and 0·6189 volt at 25°. According to this the potential of the chain at a temperature t between 18° and 25° is

$$\pi = 0{\cdot}6229 - 0{\cdot}00057\ (t - 18) \text{ volt.}$$

For the corresponding chain containing 2 N sulphuric acid Schreiner [1925] has found the constant 0·000651 between 12° and 22° and 0·000641 between 22° and 32°. These values agree so well with the factor 0·00057 that we have calculated the potentials at 18° for the phosphate mixtures examined by Grossman at 19°. Further, from our values in Table IV we have calculated by means of graphic interpolation the potentials of mixtures which have not been directly measured by us. The values are recorded in Table XII. The values for the mixtures Nos. 1 and 3 are taken directly from Table IV, whereas the values for the mixtures Nos. 2 and 4 are found by interpolation.

Table XII.

	Phosphate mixture (cc.)			
No.	1/15 M Na_2HPO_4	1/15 M KH_2PO_4	Grossman	Biilmann and Klit
1	50	50	0·6165	0·6229
2	61	39	0·6203	0·6233
3	80	20	0·6241	0·6253
4	87	13	0·6259	0·6272

It will be seen that in most cases the two series of potentials do not agree so well as might be desired for the p_H determinations. Further, we shall point out that the way in which borate mixtures act with quinol is so different from the manner in which the phosphate mixtures are affected by quinol that we do not consider that a common formula will hold good for the corrections in both cases.

Summary.

1. The simple quinhydrone electrode and the quinoquinhydrone and the hydroquinhydrone electrodes have been examined in phosphate and borate mixtures by comparison with hydrogen electrodes in the same mixtures and the potentials of chains of these types are determined for phosphate mixtures with p_H 5·90–8·04 and borate mixtures with p_H 7·34–8·05.

2. The simple quinhydrone electrode gave correct p_H values in phosphate mixtures up to 7·73 and still very good values at p_H 8·04; in the borate mixtures it gave correct values.

3. The quinoquinhydrone electrode gave correct values in phosphate mixtures up to p_H 7·38. In more alkaline phosphate mixtures and in the borate mixtures the potentials were very drifting.

4. The hydroquinhydrone electrode gave very stable potentials in the phosphate mixtures, but too low p_H values because of the acid influence of the hydroquinhydrone. The influence of the quinol was determined by means of the colloidal palladium hydrogen electrode. The influence may be eliminated by using a comparison electrode of a similar type.

5. Owing to the acid influence of quinol, corrections must be introduced in the measurements of Biilmann and Katagiri with hydroquinhydrone electrodes in phosphate and bicarbonate solutions.

6. It is shown that the acid influence of quinol may affect the measurements of Grossman in phosphate and borate mixtures in such a manner that the correction formula of Grossman may only hold for special cases.

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CVIII. THE PHOSPHATASES OF MAMMALIAN TISSUES.

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Amongst the many phosphorus compounds which are to be found in almost every tissue of the animal body, in addition to inorganic phosphate, phosphatides and nucleoproteins, there are also present phosphoric esters of an acid-soluble nature, stable in dilute acid or alkaline solution and of relatively small molecular dimensions. The amount of phosphorus present in this ester form, in most of the tissues, is considerably in excess of that present as inorganic or phosphatide phosphorus. Enzymes capable, under certain conditions, of hydrolysing these important esters are also widely distributed in the body, and in certain tissues have been assigned specific functions which if confirmed would give these catalysts a position of very great metabolic importance.

The present paper deals with (*a*) the quantitative distribution of phosphatases in mammalian tissues, (*b*) the question as to whether a specific enzyme is required for the hydrolysis of each phosphoric ester, and (*c*) the synthetic activity of the phosphatases.

A. The quantitative distribution of phosphatases in certain mammalian tissues.

Grosser and Husler [1912], Plimmer [1913, 1], Forrai [1923] and Robison [1923] have described experiments in which ground-up tissues or tissue extracts have been shown to have differing hydrolytic activity towards glycerophosphate or other phosphoric esters, but quantitatively these results can only be considered preliminary.

The determination of the quantitative distribution of any enzyme in the tissues is, for several reasons, a problem of quite a different order from that of the distribution of a particular element or compound. In the case of an enzyme, (1) it may be found impossible to remove the enzyme from the tissues without destroying its activity, (2) extracts of different tissues may contain in addition to the enzyme greater or lesser amounts of accelerating or inhibitory substances, (3) the activity of the enzyme will vary with the c_H, giving an optimum which may be different for the different tissues, (4) the functional c_H for most intracellular enzymes is unknown, so that it is not possible to compare activities at such hydrogen ion concentrations, (5) the choice of substrate is important; the "natural" substrate may be unknown.

It is impossible to avoid all these potential sources of error; we can perform the determinations neither under strictly "natural" nor strictly artificial conditions, and a compromise is inevitable.

One fortunate preliminary finding for the phosphatase activity of all tissue extracts examined is that the optimum c_H is very nearly the same, whether the "natural" esters, separated from the tissues by precipitation of the proteins, or esters of known composition such as sodium glycerophosphate, sodium hexosediphosphate or the sodium salt of guaninenucleotide are used.

As enzyme preparations, tissue hashes have been tried; also the fluids obtained by extraction of the ground-up tissue with dilute acid and alkali, and with glycerol containing various amounts of water, of acid or of alkali. Extraction with very dilute acids, or with acid glycerol, gives poor yields. The method found to be most practicable, which gives enzyme activities on the whole greater than any other method, is simple extraction of the finely ground-up tissue with chloroform water for 24–48 hours at room temperature, followed by slow filtration through cotton wool. The turbid filtrate contains almost all of the activity of the original tissue. Extracts of gastric and intestinal mucosa are prepared as follows. In the case of the rabbit or the cat, the whole intestine is washed out well with 0·9 % NaCl, then, after draining, the first 8 inches of the small intestine are taken as duodenum, the rest of the intestine as far as the caecum is then divided into two halves, the upper half is called jejunum and the lower ileum. The mucosa is scraped off with the back of a scalpel, weighed and extracted as described. With regard to accelerating or inhibiting substances, it is found that a short dialysis (18 hours) against 20 volumes of water has little effect on the activity of either kidney or duodenal extracts, although 6 days' dialysis against repeated changes of water shows some inactivation, which in the case of kidney extracts can be partially restored by the addition of a boiled extract of kidney.

For purposes of determining the relative distribution of the enzyme in the tissues the extracts are not purified further. The method of determining the relative activity has been standardised as follows. A small portion of tissue extract (0·5 to 1 cc.), representing a known weight of original tissue and brought to the optimal c_H of the enzyme, is added to a number of tubes each containing excess of the substrate (5 cc.) in glycine-NaOH buffer (5 cc.) at a short series of c_H near to the optimum, and incubated at 37° in presence of chloroform (which is found to have a negligible effect on the activity) for exactly 2 hours. The hydrolysis is stopped by the addition of trichloroacetic acid (2 cc. of 25 %) and the amount of inorganic phosphate liberated determined by the Briggs method. Controls containing substrate + buffer, and enzyme + buffer are put up at the same time. The correction for autolysis is usually very small. The number of enzyme units per g. of original tissue is then calculated, a unit being defined as the amount of enzyme, at the optimal c_H for its activity, which liberates 1 mg. P under the above conditions. For sodium glycerophosphate, or sodium hexosephosphate or the "natural"

esters of the tissues this optimum is found to be at p_H 8·8–9·1; for the sodium salt of guaninenucleotide it is usually p_H 9·0–9·2.

In Table I are shown the average values for the tissues of the rabbit, the cat and man, with glycerophosphate as substrate. In Fig. 1 the relative activity of certain tissues of the cat towards three substrates—glycerophosphate, hexosediphosphate, nucleotide—is shown. Table II shows a comparison of two methods of extraction; the optimal c_H for phosphatase activity of extracts obtained by the two methods appears to be the same.

Table I. *Average phosphatase content of the tissues. Substrate, sodium glycerophosphate.*

Figures indicate average enzyme units per g. of original tissue (wet weight).

Tissue	Animal and number examined: Rabbit (3)	Cat (3)	Man (2)
Gastric mucosa ...	0·2	1·5	0·5
Duodenal mucosa ...	15·2	27·5	7·7
Jejunal mucosa ...	30·7	29·7	11·5
Ileal mucosa	16·1	23·9	13·6
Colon mucosa	5·3	10·0	3·7
Liver	3·6	1·1	0·8
Lung	3·2	7·6	1·0
Kidney	10·5	14·1	4·8
Spleen	5·6	0·9	1·0
Pancreas	—	0·7	—
Parotid gland	—	1·7	—
Suprarenal gland ...	—	—	1·3
Brain	1·0	0·8	0·6
Cardiac muscle ...	0·3	0·2	—
Skeletal muscle ...	0·2	0·2	—
Bone	6·0	3·0	—
Artery	—	Nil	0·1

Table II. *Two methods of extraction compared.*

Units phosphatase per g. of tissue.

Tissue	Rabbit, Tissues extracted with: Chloroform water	60 % glycerol	Man, Tissues extracted with: Chloroform water	60 % glycerol
Gastric mucosa	0·2	0·2	0·6	0·5
Duodenal mucosa ...	8·7	8·7	12·0	7·0
Jejunal mucosa	21·0	13·5	13·3	Lost
Ileal mucosa	11·8	12·2	19·9	16·5
Colon mucosa	5·6	6·4	—	—
Liver	3·8	3·7	0·7	0·7
Lung	2·5	4·6	0·8	0·6
Kidney	12·6	11·8	3·4	3·3
Spleen	4·5	5·1	1·3	0·8
Brain	1·4	1·5	0·3	0·4
Heart	0·3	0·3	0·2	0·2
Muscle	0·2	0·2	—	—

There is a surprising similarity between the distribution in animal tissues of glycerophosphatase (or hexosephosphatase or nucleotidase) and that of ereptase, the enzyme investigated by Vernon [1904, 1905], which hydrolyses

peptones to substances which do not give the biuret test. Two examples of this similarity are given in Tables III and IV[1].

Table III. *Enzyme distribution in cat's tissues.*

Tissue	Enzyme: Glycerophosphatase Mean of 3	Hexosediphosphatase One individual	Ereptase (Vernon) Mean of 2
Gastric mucosa ...	1·5	2·2	3·9
Duodenal mucosa	27·5	53·3	27·7
Jejunal mucosa ...	29·5	45·7	18·2
Ileal mucosa ...	23·9	24·6	14·4
Colon mucosa ...	10·0	11·7	5·8
Liver	1·1	1·6	5·0
Lung	7·6	[3·8]*	6·9
Kidney	14·1	22·9	14·3
Spleen	0·9	2·2	7·6
Pancreas	0·7	—	6·4
Brain	0·8	[1·0]*	1·2
Cardiac muscle ...	0·2	1·5	1·6
Skeletal muscle ...	<0·2	0·4	0·8

* From a second cat.

Table IV. *Phosphatase and ereptase in the kidneys of various animals.*

	Units (average): Rabbit	Cat	Man	Guinea-pig
Ereptase (Vernon)	[8] 10·9	[8] 11·6	[2] 5·2	[7] 8·8
Glycerophosphatase	[5] 11·4	[3] 14·1	[11] 4·8	[1] 7·9

Figures in brackets show number of individuals.

Phosphatase and ereptase also appear to be extractable from the tissues in the same way, to have their optimal activity in the same region of hydrogen ion concentration, and to have similar stabilities. In addition there are also remarkable correspondences (*a*) between the amount of ereptase and phosphatase in various organs at different stages in foetal and post-natal life (compare Vernon [1905] and figure given by Kay [1926, 1]) and (*b*) between the amounts in healthy and in diseased organs (Table V).

Table V. *Enzyme activity in diseased and healthy kidneys.*

	Phosphatase units [Brain and Kay, 1927] Average	Ereptase units [Vernon, 1908] Average
Healthy kidneys	[11] 4·8	5·4
Parenchymatous degeneration	[7] 3·7	3·4
Advanced nephritis	[9] 1·6 to 0·4	2·8 to 0·86

Figures in brackets show number of individuals.

The significance of this curious correlation between the enzyme hydrolysing phosphoric esters and the enzyme hydrolysing peptones is difficult at present to evaluate, but points to some functional relationship in the cell between these apparently unconnected activities.

[1] By accident the numerical values of the units by which the activities of the two enzymes glycerophosphatase and ereptase are expressed are almost the same.

B. Possible identity of certain phosphoric esterases.

Extracts of various tissues, prepared as just described, will hydrolyse a number of naturally occurring, and of synthetic phosphoric esters. Is the agent responsible for these hydrolyses the same, or is there a specific phosphatase for each substrate?

There is a good deal of cumulative evidence in favour of the identity of the glycerophosphatase with the hexosediphosphatase (at least in the liberation of the first half of the phosphoric acid) and with the nucleotidase of mammalian tissues. This evidence is as follows.

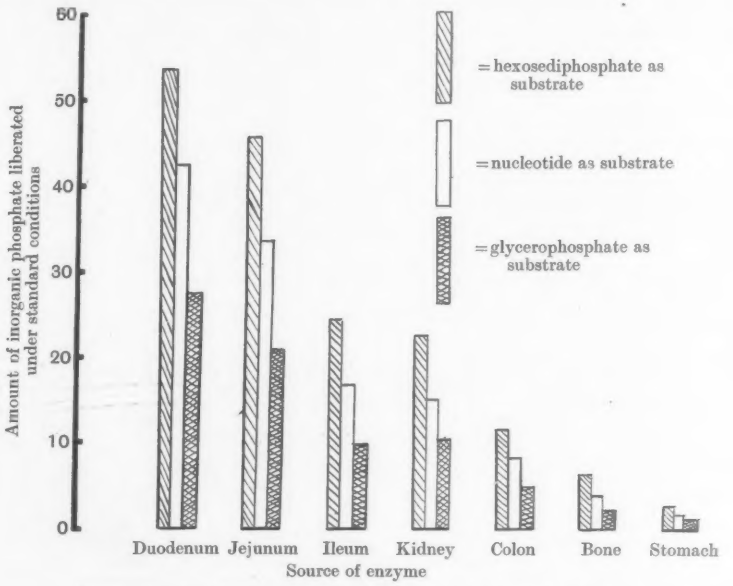

Fig. 1. Possible identity of glycerophosphatase, hexosediphosphatase and nucleotidase.

(*a*) *Optimal* c_H *for hydrolysis.* If a tissue preparation attacks different substrates at a different optimum c_H, it does not necessarily follow that different enzymes are concerned. But if a tissue preparation attacks closely related substrates at the same optimum c_H, there is a strong likelihood that the same enzyme is responsible. It has already been shown [Kay, 1926, 2] that the hydrolysis of both sodium hexosediphosphate and sodium glycerophosphate by kidney phosphatase has the same optimum c_H. The same optimum c_H has since been found for the action of the various intestinal extracts and of lung, liver and bone phosphatase on these two substrates, though usually the optimum c_H for glycerophosphate hydrolysis by these

extracts is at about p_H 9·0, and for hexosephosphate at about p_H 9·1. With the same extracts guaninenucleotide (pure specimen prepared by the method of Buell and Perkins [1927] from yeast nucleic acid) is hydrolysed at an optimum p_H of about 9·2. These optima are thus sufficiently near to one another to arouse the suspicion that the enzymes concerned are not very dissimilar.

(*b*) Using extracts from various tissues to hydrolyse the same three substrates under the same conditions (of organic phosphorus concentration per cc., time, temperature, quantity of enzyme) it is found that there is a constant ratio between the amounts of inorganic phosphate liberated from the three substrates by any one of these extracts. This is sufficiently well indicated by Fig. 1 to require few further remarks. A similar ratio holds for human and rabbit tissues as for those of the cat (from which the values shown in Fig. 1 are derived), and this same ratio holds good within a small experimental error whether the tissue extracts are made with chloroform water or with 60 % glycerol.

(*c*) If two of the above substrates in the same solution are together exposed to the action of *small quantities* of such tissue extracts, the total liberation of inorganic phosphate is considerably less than what would be expected if two independent enzyme systems were present. It is, of course, essential that only small quantities of the hydrolysing agent be used, in order to keep the enzyme working at maximal activity during the period of the experiment. In Table VI are given the results of a few such experiments.

Table VI. *Identity of glycerophosphatase, hexosediphosphatase and nucleotidase.*

P in mg. liberated in 2 hours at 37·5°.

	Enzyme used, extract of			
Substrate used*	Cat's duodenum	Rat's kidney	Rat's bone	Cat's duodenum
Glycerophosphate	0·064	0·058	0·169	0·304
Hexosediphosphate	0·136	0·120	0·246	—
Nucleotide	—	—	—	0·299
Two together:				
(*a*) found	0·132	0·089	0·180	0·301
(*b*) calculated if two independent enzymes present	0·200	0·178	0·415	0·603

* The organic P concentration of the substrates is not necessarily the same.

(*d*) If sodium fluoride is added to the reaction mixture containing a phosphatase and either glycerophosphate or hexosediphosphate, there is less hydrolysis during a given time than in the absence of this salt. Sodium glycerophosphate and sodium hexosediphosphate of the same original concentration of organic phosphorus have been hydrolysed by the same tissue extract in presence of increasing amounts of sodium fluoride, and it has been found that the percentage inhibition runs approximately parallel in both cases. With both substrates the effect of sodium fluoride may be just detected in as

low a concentration as $M/100$, the inhibition is 25–35 % in $M/25$ and 60–70 % in $M/6$ fluoride. The fact that an inhibiting agent interferes with the hydrolysis of both phosphoric esters at about the same rate is another small piece of evidence in favour of the identity of the two enzymes concerned. Sodium fluoride also inhibits the hydrolysis of nucleotide, but comparative quantitative experiments have not been made.

György [1925] has shown that phosphatolysis in all the tissues he examined (blood, liver, kidney, brain, heart muscle) appears to have a similar mechanism. It is stimulated by hydroxyl, by a low concentration of lactate or by potassium ions, checked by hydrogen, calcium and (a high concentration of) lactate ions.

It is submitted that although each of the foregoing points is, by itself, far from sufficient proof of the identity of the three enzymes, yet taken together they make a strong case in favour of such identity.

C. Synthetic activity of the phosphatases.

To the short list of enzymes proved to be capable of synthetic activity must be added tissue phosphatase. Previous workers (Bodnár [1925], Martland and Robison [1927]) have shown that under certain conditions inorganic phosphate disappears in presence of tissue extracts derived either from the plant or the animal. Embden and Zimmermann [1924] have isolated hexosediphosphoric acid from the reaction mixture after allowing muscle press juice to act at low temperature (10°) on glycogen plus inorganic phosphate in presence of sodium fluoride. The reaction is evidently a complex one. So far this is the only case on record in which a phosphoric ester has been isolated following the synthetic enzymic activity of a tissue extract.

Using tissue extracts prepared as described earlier in the present paper, the enzymic synthesis of certain phosphoric esters can be accomplished with great ease at 37° and at p_H 8–9, provided the alcohol concerned is present in sufficiently large excess in the reaction mixture. The balanced reaction

$$\text{alcohol} + \text{acid} \rightleftharpoons \text{ester} + \text{water}$$

is thus pushed over toward the right hand side (the alcohol concentration being high and constant and the effective water concentration low). Synthesis goes on until an equilibrium is reached, the position of which depends in practice mainly on the amount of alcohol present.

Preliminary experiments have been carried out by mixing sodium phosphate solutions at p_H about 8·4 with duodenal or kidney extracts, and adding various alcohols or strong aqueous solutions of alcohols to the mixture. A sample of the reaction mixture is then taken at once, the proteins precipitated and inorganic and total phosphorus determined in an aliquot portion of the filtrate. The reaction mixture is raised to 37·5° and at intervals samples are taken and their content of inorganic and total phosphorus determined. (Controls using boiled enzyme are invariably used, and in no case show appreciable change in the concentration of inorganic phosphate.)

By this means the following facts have been established:

(*a*) Both kidney and intestinal extracts are capable of esterifying phosphoric acid in presence of high concentration of methyl or ethyl alcohol, ethylene glycol or glycerol.

(*b*) With propyl alcohol and dextrose, although definite synthesis has been observed, the amounts so synthesised have been very small.

(*c*) The products formed from glycerol (and glycol) were stable to 1 % sodium hydroxide at 100° for 4 hours at least. This is characteristic of glycerophosphates (Plimmer [1913, 2]).

(*d*) By simply diluting a portion of the reaction mixture with water, partial hydrolysis of the synthesised ester was usually observed.

(*e*) Sodium fluoride diminishes the *rate* of synthesis as of hydrolysis but probably does not affect the position of equilibrium. A typical experiment is shown in Table VII.

Table VII. *Effect of sodium fluoride on synthetic activity of phosphatase.*

Tube *A*. 1 cc. Na_2HPO_4 solution at p_H 8·4; 2 cc. cat's duodenal extract; 5 cc. glycerol, 2 cc. water.
Tube *B*. As *A*, but 1 cc. 0·8 *M* NaF (at p_H 8·4) and 1 cc. water in place of 2 cc. water.
Tube *C*. As *A*, but 2 cc. 0·8 *M* NaF in place of 2 cc. water.
Tube *D*. As *A*, but enzyme first boiled.
Tubes *E*, *F*, *G*, *H*, as tubes *A*, *B*, *C*, *D*, but using kidney extract from same cat.

	Percentage synthesis							
	Duodenal extract				Kidney extract			
Time (days after start)	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1	17	16	11	0	5	5	3	0
4	26	23	15	0	20	18	10	0
13	26	24	18	0	24	23	14	0
37	25	23	21	0	24	22	18	0

The above table also shows that the same position of equilibrium is reached with the extracts derived from two quite different tissues as catalysts.

The time relationships of the balanced reaction.

$$Na_2HPO_4 + C_3H_5(OH)_3 \overset{\text{phosphatase}}{\rightleftharpoons} C_3H_5(OH)_2PO_4Na_2 + H_2O$$

have been studied. Two solutions are made up having the same amount of total P per cc.; in one, however, all the phosphorus is present as inorganic phosphate, in the other, all as sodium β-glycerophosphate, the p_H of each being 8·9. To each is added an equal volume of duodenal extract at p_H 8·9 (previously dialysed for 18 hours) and twice the volume of neutral glycerol. Controls with inactivated enzyme are also made up.

The results are shown in Fig. 2, curves *A*, *A*. Curves *B*, *B* represent the results of a similar experiment in which the reaction has taken place in 75 % glycerol (by volume). In both experiments a further determination (not shown in the figure) has been made on the reaction mixtures after 6 days without showing significant change in the state of equilibrium.

From Fig. 2 it is clear that the same equilibrium position is reached whether inorganic sodium phosphate or sodium glycerophosphate is present to begin with, provided, of course, that water and glycerol are present in

amounts which are large relative to the amount of phosphorus (the concentration of P was only 22 mg. per 100 cc. of the reaction mixture). The position of the equilibrium, and the speed at which it is reached, are also seen to be dependent on the relative concentrations of glycerol and water.

Curves similar to those of Fig. 2 have also been obtained using kidney phosphatase as the catalyst.

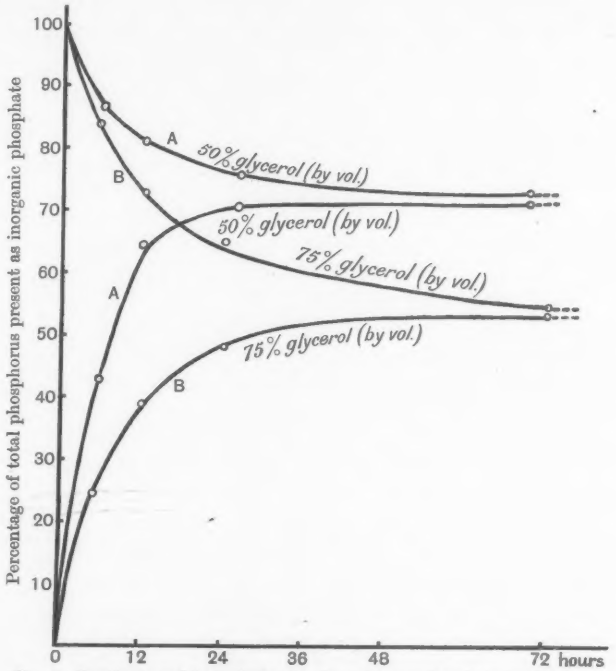

Fig. 2. Synthesis and hydrolysis of sodium glycerophosphate by duodenal phosphatase.

Inhibitory effect of free phosphate.

Free phosphate, even in relatively low concentrations is found to have a marked inhibitory effect on the hydrolysis of glycerophosphate by kidney or duodenal phosphatase (Table VIII).

Table VIII. *Inhibition of phosphatase by inorganic phosphate.*

Concentration of glycerophosphate at start	Concentration of inorganic phosphate at start	% inhibition after 2 hours' hydrolysis
M/40	Nil	0
,,	*M*/400	4·6
,,	*M*/200	12
,,	*M*/133	30
,,	*M*/100	62

Although low concentrations of phosphate thus markedly inhibit hydrolysis, free glycerol in much larger concentrations (up to about $M/2$) has no such effect. Since the phosphatase is clearly specific, not for the alcohol portion, but for the phosphoric acid portion of the molecule (which is the only part common to all the esters) one would expect the phosphate configuration to be in close relationship with the structure of the enzyme molecule, and the finding that the activity of the enzyme is very sensitive to the presence of inorganic phosphate is not surprising.

Isolation of products of synthesis by phosphatase.

Sodium glycerophosphate has been isolated from the reaction fluid after allowing duodenal extracts to act on a mixture of sodium phosphate and glycerol for a week.

Method. 10 g. of Na_2HPO_4, $12H_2O$ were dissolved in 100 cc. of extract of cat's duodenal mucosa (1 in 20) which had stood at room temperature in presence of chloroform for a week, and contained only traces of organic phosphorus. To this solution were added 160 cc. of pure glycerol. A sample of the liquid was taken at once for free and total P determination, and further samples at intervals afterward.

Time (days)	Free P (mg. per cc. liquid)	Total P	Organic P
0	3·60	3·61	0·01
4	2·91	—	0·70
7	2·39	—	1·22
10	2·36	3·66	1·30

After 10 days the reaction mixture was heated to 100° for 10 minutes to inactivate the enzyme, then diluted with an equal volume of water, and $Ca(NO_3)_2$ added in slight excess followed by ammonia till no more precipitate was obtained. The mixture stood for a short time and was then filtered. The filtrate (500 cc.) contained no free phosphate, but 200 mg. organic phosphorus. It was boiled to remove ammonia, neutralised to litmus, and excess of lead acetate (25 %) added. After standing overnight the mixture was centrifuged. The supernatant liquid now contained no free phosphorus, but 14 mg. of organic phosphorus. After washing the precipitate twice with water it was resuspended in warm water and the lead removed by H_2S. The filtrate from lead sulphide now contained 6 mg. free P and 180 mg. organic P. 5 g. barium acetate were dissolved in it, and baryta was added until the reaction was alkaline to phenolphthalein. After filtering off the barium phosphate, barium glycerophosphate was precipitated by adding 4 volumes of alcohol. This process was repeated twice. The resulting product was dried *in vacuo* at 100° over phosphorus pentoxide. It weighed 1·60 g.

Found:	Ba, 44·5 % 44·6 %	P, 9·9 %
Calculated for $C_3H_7O_6PBa$	Ba, 44·7 %	P, 10·1 %.

A portion dissolved in water was precipitated by the exact quantity of sodium sulphate, and the filtrate found to contain organic phosphorus readily hydrolysed by kidney phosphatase but quite stable to 1 % NaOH at 100° for 6 hours. A portion of the barium salt gave the acrolein test for glycerol. The isolated salt was therefore the Ba salt of glycerophosphoric acid. Both the free acid and the barium salt in aqueous solution were optically inactive.

There is a distinction to be drawn between true phosphatases and other enzymes which are phosphatases incidentally. The true phosphatases, whose point of attack is the phosphoric acid portion of the ester molecule, are relatively stable enzymes. Tissue extracts kept at 0–4° in presence of chloroform retain their ability to hydrolyse phosphoric esters for a long time. One specimen of pig's kidney extract, which had an initial content of 0·31 unit per cc., had after 8 months a content of 0·26 unit, and other specimens have been kept for even longer periods without serious loss of activity. Dried tissues keep their phosphatase activity almost indefinitely. In muscle extracts prepared by the method described above there is very little of the true phosphatase. The activity towards glycerophosphate is practically nil, although there is distinctly more activity if hexosediphosphate is used as substrate. The difference is more marked with hashed muscle which hydrolyses hexosephosphate fairly readily but hardly attacks glycerophosphate. In the case of muscle extracts therefore the ratio between P liberated from hexosephosphate and from glycerophosphate by the same extract is anomalous and very different from the figure obtained under the same conditions using kidney, bone or intestinal extracts. Meyerhof [1926] found that an unstable enzyme could be separated from frog's or rabbit's muscle which hydrolysed glycogen and other polysaccharides to lactic acid, and also hydrolysed hexosephosphate, even after warming to 38°. Lactic acid being produced in the latter case also, the point of attack of this enzyme on the hexosephosphate molecule is clearly the alcohol radicle. It is probable that this explains the anomaly, the muscle extracts prepared by the ordinary extraction method still containing small quantities of the unstable *pseudo*-phosphatase. The true phosphatase appears not to produce lactic acid from naturally occurring phosphoric esters. The present author in conjunction with Dr J. T. Irving has recently found that kidney phosphatase will not produce lactic acid from the acid-soluble phosphoric esters of the kidney tissue, nor apparently from sodium hexosediphosphate[1].

Meyerhof [1927] believes that the formation of a phosphoric ester is necessary before lactic acid can be formed from polysaccharides. Possibly one function of the true phosphatases *in vivo* is the synthesis of such esters. This possibility is supported by the experiments described in section C. There is no reason to believe that such synthetic activity would be limited to simple

[1] Duodenal phosphatase also hydrolyses fermentation hexosediphosphate without production of lactic acid. An extract of cat's duodenal mucosa hydrolysed 90 % of a solution of hexosediphosphate in 6 hours, producing 0·82 mg. inorganic P per cc., without producing any detectable quantity of lactic acid (*i.e.* less than 0·01 mg. per cc.).

esters. Since the phosphatases hydrolyse nucleotides (*in vitro*) with great ease, it is possible that under controlled conditions in the cell they are able to catalyse the synthesis of these phosphoric esters from nucleosides. This has not yet been attempted *in vitro*. Tissue phosphatases may similarly enter into the early stages in the formation of phosphatides. Further experiments on the synthetic activity of these enzymes are in progress.

It is nevertheless well established that *in vivo* the phosphatases may act hydrolytically. If sodium hexosephosphate or glycerophosphate be administered to an animal either intravenously or by mouth, the inorganic phosphate content of the blood and of the urine rises rapidly.

SUMMARY.

1. The phosphatases of mammalian tissues (*i.e.* the relatively stable enzymes present in extracts from such tissues which hydrolyse acid-soluble phosphoric esters) all appear to have an optimum p_H between 8·8 and 9·3. The character of the substrate influences this optimum to a slight extent. The distribution of phosphatase in the tissues of the rabbit, of the cat and of man has been quantitatively determined. This distribution is remarkably akin to that found for ereptase by Vernon. Other correspondences between these functionally widely dissimilar enzymes are described.

2. It is probable that the same enzyme is responsible for the hydrolysis of glycerophosphate, of hexosediphosphate and of nucleotide.

3. In presence of excess of the alcohol concerned, inorganic phosphate can be esterified, using phosphatases derived from various tissues as catalysts. Glycerophosphoric acid (isolated as the barium salt) has been synthesised in this way from glycerol, and several other alcohols have been shown to combine with inorganic phosphate. The equilibrium

$$\text{glycerol} + \text{phosphate} \rightleftharpoons \text{glycerophosphate} + \text{water}$$

has been studied, using phosphatase as catalyst.

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CIX. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

I. THE RELATIONSHIP BETWEEN PHOSPHORIC ESTER ACCUMULATION AND PHOSPHORIC ESTER BREAKDOWN AND LACTIC ACID FORMATION FROM GLYCOGEN.

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(*Received April 4th, 1928.*)

The experiments recorded in this communication are part of an investigation into lactic acid and phosphoric ester formation from carbohydrates in muscle extract. The particular experiments recorded below deal with the supposed relationship of a quantitative nature between the lactic acid formation and the phosphoric ester accumulation and breakdown that accompany that process. Gottschalk [1926] gives a résumé of this subject and sets forth a series of reactions to explain the experimental results of Meyerhof. These results showed that in lactic acid formation from glycogen in muscle extract there are two distinct periods. In the first period there occurs a high rate of lactic acid formation and an accumulation of phosphoric ester. The extent of this accumulation is conditioned by the amount of carbohydrate or the amount of available phosphate. In the second period this accumulated ester is broken down and the rate of lactic acid formation diminishes. It is postulated that in the first period, for every two molecules of diphosphoric ester formed, one is immediately broken down to the end-product, lactic acid, while the other is "stabilised" and accumulates. This implies that for every two molecules of lactic acid formed, two molecules of inorganic phosphate are retained as ester, giving a molar ratio of lactic acid to phosphate retained as ester of unity. Since it is also put forward that the lactic acid formed in the second period originates from the ester accumulated in the first period, the molar ratio of lactic acid to increase in inorganic phosphate should again be unity.

It is to this aspect of the problem that the following experiments apply. Attention is drawn to the fact that the extracts employed here are, relatively speaking, fresh and concentrated. These extracts show different phenomena from those that have been kept for a short time at 37° prior to the addition of glycogen. This will be made the subject of a subsequent communication.

Experimental procedure.

Cats were killed by a blow on the back of the head and were immediately perfused until free from blood, first with warm and then with cold Ringer's solution. The excised muscles were minced and then put through a grinding mill. The muscle pulp was ground up with silver sand in a mechanical mortar in lots of 350 g. A solution containing sodium bicarbonate and sodium chloride, both to the extent of 0·5 % and cooled to freezing point, was added in portions until about 350 cc. had been added in all. From the mortar the mixture was poured into a sheet of muslin and the extract was squeezed out in a hand-press. The combined extract was then frozen with stirring and filtered through a Büchner funnel without paper, thus separating the extract from the ice crystals. Repetition of this process soon reduces the volume to any desired extent. As a rule it took about 90 minutes to get the extract ready for incubation but every precaution was taken to keep the temperature low.

Before incubation more phosphate was added to the extent of 5 cc. of a 3 % solution of NaH_2PO_4 per 100 cc. extract. The reaction was adjusted if necessary to about p_H 7·2 by the addition of a saturated solution of sodium bicarbonate. In the experiments described below, the extract was divided up into lots of 160 cc. and placed in the incubation flasks. As 5 cc. were required for phosphate estimation and 10 cc. for the lactic acid estimation, one required for every withdrawal, at the appointed intervals, about 20 cc. The requisite volume of a freshly prepared 10 % solution of glycogen was measured out into very small flasks, water being added in the cases of the lower concentrations in order that the volume of fluid added might be the same in every case. The contents of the incubation flask were rapidly warmed to 37°, the glycogen solution was added and the mixture thoroughly shaken up. First, 5 cc. were pipetted out into 25 cc. of 2 % trichloroacetic acid and the mixture was at once frozen solid. It was kept in this condition until the phosphate analysis was done. Immediately after this 10 cc. were pipetted into a dry flask and 30 cc. of a tungstic acid solution added. (This tungstic acid was prepared just before the precipitation by mixing 10 cc. of a 5 % solution of sodium tungstate and 25 cc. of 0·5 *N* sulphuric acid.) The time was noted by an observer and the incubation flask immersed in a large water-bath at 37°. Each flask was fitted with a stopper holding three tubes. One reached to the bottom of the flask and through this was passed a stream of nitrogen, previously bubbled through a pyrogallol-potassium hydroxide solution. The second tube was a short elbow tube, not dipping into the extract, and served as a gas outlet. The third tube, like the first, dipped well under the extract; by closing the gas inlet by compressing the rubber connection and blowing gently through the gas outlet the extract could be siphoned out of the flask when it was desired to take a sample. If the second sample was to be taken 10 minutes after the start of the incubation only four incubation flasks could be used simultaneously with comfort, allowing for the time required for warming up the extract and for the pipetting. The

times of withdrawal were indicated by an assistant and were correct to a quarter of a minute.

The inorganic phosphorus was estimated by the method of Fiske and Subbarow [1925]. The colour was allowed to develop for 20 minutes in order to convert into inorganic phosphorus the whole of the labile phosphorus of phosphagen which was therefore included in the determinations of "inorganic phosphorus." The term "inorganic" phosphorus is thus really a misnomer, but as it was intended to investigate lactacidogen formation and breakdown, decrease and increase of "inorganic" phosphorus as estimated by the above method can be taken as indication of lactacidogen accumulation and breakdown. Under the above conditions lactacidogen and hexosediphosphoric acid are stable.

The lactic acid was estimated by the method of Clausen [1922], taking advantage of the improvements suggested by Shaffer [1927]. Interfering carbohydrates were removed by the usual copper-lime method. 20 cc. of the tungstic acid filtrate were accurately pipetted into a 50 cc. volumetric flask, 10 cc. of a 7 % lime suspension added, followed by 6 cc. of a 7 % copper sulphate solution. After shaking at intervals for half an hour, 2 cc. more of the lime suspension were added and the volume made up to 50 cc. This gives a final dilution of the extract of 1 in 10. 10 cc. of the filtrate of this were used for the oxidation by 0·005 *N* $KMnO_4$. The aldehyde was aerated into two tubes, the first holding 30 cc. of 0·5 *N* bisulphite and the second 20 cc. The excess of the unbound sulphite was titrated first with 0·1 *N* iodine and finally with 0·005 *N* iodine until just blue. Clausen underestimates the error incurred by overstepping this first end-point. The bound sulphite was titrated, with the gradual addition of saturated sodium bicarbonate solution, with accurate 0·005 *N* iodine. The results are based on a 95 % yield of acetaldehyde.

Experimental results.

The values for lactic acid and for "inorganic" phosphorus are expressed in the following tables as milligram-molecules or milligram-atoms respectively per 100 cc. extract multiplied by 100. Thus a concentration of 90 mg. lactic acid is represented by 100 as is also a concentration of "inorganic" phosphorus of 30·96 mg. per 100 cc. extract. A change of one unit in the tables indicates, for lactic acid, a change in concentration of 0·9 mg. and for phosphorus 0·3 mg. per 100 cc. extract. The figures can thus be used as molar quantities in any calculations.

Exp. 237.

	A. Glycogen 0·4 %		B. Glycogen 1·0 %	
	Extract 200 cc. 10 % glycogen 10 Water 15		Extract 200 cc. 10 % glycogen 25	
Time	Lactic acid	Phosphorus	Lactic acid	Phosphorus
Start	139	163	139	165
10 mins.	212	143	244	100
20 „	275	113	304	45
30 „	319	43	337	18
40 „	335	41	354	24
50 „	349	49	373	35
60 „	373	67	397	50
90 „	402	102	447	97

Exp. 240.

	A. Glycogen 0·3 %		B. Glycogen 0·6 %		C. Glycogen 0·9 %	
	Extract 160 cc.		Extract 160 cc.		Extract 160 cc.	
	10 % glycogen 5		10 % glycogen 10		10 % glycogen 15	
	Water 19		Water 14		Water 9	
Time	Lactic acid	Phosphorus	Lactic acid	Phosphorus	Lactic acid	Phosphorus
Start	214	176	214	177	221	177
10 mins.	264	124	267	98	273	70
20 „	293	113	283	24	291	17
30 „	317	107	299	28	304	25
40 „	340	122	315	48	321	43
50 „	363	148	333	68	329	69
60 „	371	165	344	83	351	91
90 „	381	200	356	164	366	171

Exp. 241.

	A. Glycogen 0·4 %		B. Glycogen 1·0 %	
	Extract 160 cc.		Extract 160 cc.	
	10 % glycogen 8		10 % glycogen 20	
	Water 24		Water 12	
Time	Lactic acid	Phosphorus	Lactic acid	Phosphorus
Start	222	180	220	190
10 mins.	289	122	309	86
20 „	330	150	370	93
30 „	373	152	421	116
40 „	412	156	464	132
50 „	452	169	518	145
60 „	473	191	577	149
90 „	480	218	707	154

Period of ester accumulation.

As the results show, the period of ester accumulation is a very variable quantity both in extent and duration. Exps. 237, 240 and 241 are considered in detail. The increase in lactic acid and the decrease in phosphorus are given for each interval examined and by dividing the former by the latter, the molar ratio of lactic acid formed to organic phosphorus accumulated as ester at the end of each period is obtained.

Exp. 237.

Time interval (mins.)	Glycogen 0·4 % L.A.	Phos.	L.A./Phos.	Glycogen 1·0 % L.A.	Phos.	L.A./Phos.	L.A.	Phos.	L.A./Phos.
0–10	+73	−20	3·65	+105	−64	1·61			
10–20	+63	−30	2·10	+ 60	−55	1·09			
20–30	+44	−70	0·51	+ 33	−27	1·22			
0–30	+180	−120	1·50	+198	−147	1·35			

Exp. 240.

	Glycogen 0·3 %			Glycogen 0·6 %			Glycogen 0·9 %		
0–10	+50	−52	0·96	+53	−79	0·68	+52	−107	0·49
10–20	+29	−11	2·63	+16	−74	0·22	+18	− 53	0·34
0–20	+79	−63	1·25	+69	−93	0·74	+70	−160	0·44

Exp. 241.

	Glycogen 0·4 %			Glycogen 1·0 %		
0–10	+67	−58	1·16	+89	−104	0·86

These three experiments were made with extracts prepared in exactly the same manner from three different animals. The comparison between the low and the high concentrations of glycogen with the same extract indicates that

above a certain concentration of glycogen, ester accumulation is more powerfully influenced by the glycogen concentration than is the lactic acid formation. In Exp. 240 this is particularly so. In the first 10 minutes the lactic acid formation is practically the same for concentrations of 0·3, 0·6 and 0·9 % of glycogen, while, on the other hand, the ester accumulation with 0·9 % glycogen is double that with 0·3 %. With one exception, increasing the glycogen concentration leads to a decrease of the ratio of lactic acid to phosphorus accumulated as ester, by increasing the latter quantity out of proportion to the former.

Period of breakdown of the accumulated ester.

Analyses as above are now given for the second period with the same three experiments, the lactic acid formation and the phosphorus increase for every interval being compared.

Exp. 237.	Glycogen 0·4 %			Glycogen 1·0 %					
Time interval (mins.)	L.A.	Phos.	L.A./Phos.	L.A.	Phos.	L.A./Phos.	L.A.	Phos.	L.A./Phos.
40–50	+14	+ 8	1·75	+19	+11	1·73			
50–60	+24	+18	1·33	+24	+15	1·80			
60–90	+29	+35	0·83	+50	+47	1·06			
40–90	+67	+61	1·09	+93	+73	1·27			

Exp. 240.	Glycogen 0·3 %			Glycogen 0·6 %			Glycogen 0·9 %		
30–40	+23	+15	1·53	+16	+20	0·80	+17	+18	0·94
40–50	+23	+26	0·80	+18	+20	0·90	+ 8	+26	0·30
50–60	+ 8	+17	0·47	+11	+15	0·73	+22	+22	1·00
60–90	+10	+35	0·28	+12	+81	0·15	+15	+80	0·19
30–90	+64	+93	0·69	+57	+136	0·42	+62	+146	0·42

Exp. 241.	Glycogen 0·4 %			Glycogen 1·0 %		
10–20	+41	+28	1·48	+61	+ 7	8·71
20–30	+43	+ 2	21·5	+51	+23	2·21
30–40	+39	+ 4	9·9	+43	+16	2·69
40–50	+40	+13	3·07	+54	+13	4·15
50–60	+21	+22	0·95	+59	+ 4	14·7
60–90	+ 7	+27	0·26	+130	+ 5	26·0
10–90	+191	+96	2·0	+398	+68	5·85

In this period the above ratio is a very variable quantity. The onset of the breakdown of the ester accumulated in the first period is not in every case due to lack of glycogen but occurs in the presence of excess of glycogen. As a general rule, well demonstrated in Exp. 241, the value of the ratio diminishes as the glycogen concentration falls. When all the glycogen has been used up the ratio is invariably less than unity.

Two special cases are now given. The results of Exp. 239 are given in the accompanying graphs. Fig. 1 indicates the changes in "inorganic" phosphorus and Fig. 2 the changes in lactic acid both at intervals of 10 minutes, expressed in the same units as above. The peculiar feature here is the very slight accumulation of ester in the first 10 minutes with all three concentrations of glycogen but followed in the case of 1·0 % glycogen by a second

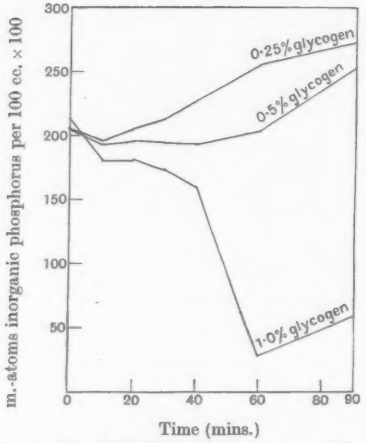

Fig. 1. Exp. 239. Phosphorus changes with glycogen concentrations of 0·25 %, 0·5 % and 1·0 %.

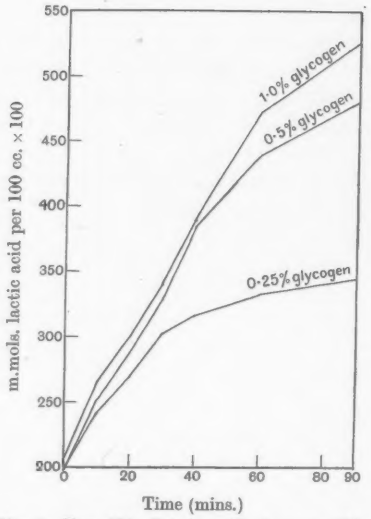

Fig. 2. Exp. 239. Lactic acid changes with glycogen concentrations of 0·25 %, 0·5 % and 1·0 %.

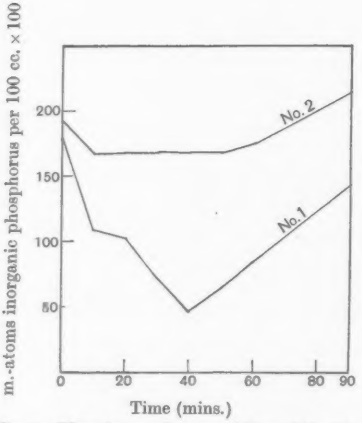

Fig. 3. Phosphorus changes of Exp. 243. No. 2 extract more concentrated than No. 1.

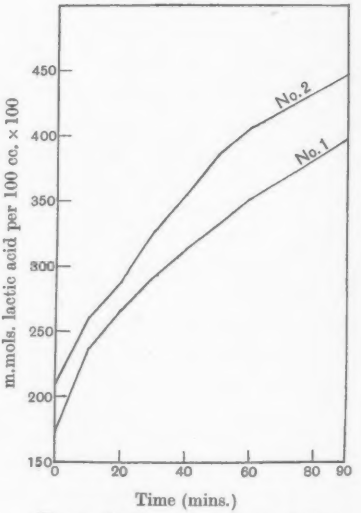

Fig. 4. Lactic acid changes of Exp. No. 243.

period of extensive ester accumulation. Contrasting 0·5 % and 1·0 % glycogen, it is shown that despite the great difference in behaviour of the phosphorus curves in the last 50 minutes, the lactic acid curves in these two cases are not very markedly different and the great ester accumulation with 1·0 % glycogen is not reflected in the corresponding lactic acid curve as it would be if these two processes were intimately related. Again, the difference between 0·25 % and 0·5 % glycogen is not so great in the phosphorus curves as it is in the lactic acid curves.

This same result was produced in Exp. 243 in the following manner. The muscles from two cats were mixed. Of the combined weight, 500 g. were extracted and gave 650 cc. extract, which was reduced by freezing to 450 cc. This is extract No. 1. The remaining 1200 g. muscle were extracted with a relatively smaller volume of extracting solution and yielded a litre of extract. This extract, No. 2, was not frozen at all. The p_H of both was adjusted to 7·3. The glycogen concentration in both cases was 0·3 %. The results are shown in Figs. 3 and 4. The outstanding feature here is the absence of ester accumulation in No. 2 extract. In No. 1 extract, on the other hand, ester accumulation is quite decided. On comparing the lactic acid curves it is seen that a high rate of lactic acid formation is not conditioned by, or related to, ester accumulation. This experiment demonstrates the danger of generalising from the results of extract No. 1, when by a slight alteration in the method of preparing the extract from the same batch of muscle, the results of No. 2 can be produced.

SUMMARY.

In the type of extract of cat muscle used in this investigation it has been found that phosphoric ester accumulation is not an essential accompaniment of lactic acid formation from glycogen. When ester accumulation does occur, there is no definite constant ratio of the molar amount of lactic acid produced to the molar amount of phosphorus that accumulates as ester. In the second period, when the ester that accumulates in the first period is broken down, the molar ratio of lactic acid produced to phosphorus set free is also very variable.

In concluding, the author would like to express his thanks to Prof. Waymouth Reid, F.R.S., for undertaking the perfusion of the animals.

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CX. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

II. THE EFFECT OF SODIUM HEXOSEDIPHOSPHATE ON THE RATE OF ESTER ACCUMULATION DURING THE INCUBATION OF GLYCOGEN IN CERTAIN TYPES OF EXTRACTS.

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(*Received April 4th, 1928.*)

In the first paper of this series it was stated that by a modification of the conditions in an extract, the balance between ester formation from glycogen and the co-existing breakdown of that ester could be changed. One method of producing this is by adding sodium hexosediphosphate along with the glycogen, and this method is described in detail in this paper.

The idea that prompted these experiments was the following. Sodium hexosediphosphate when incubated alone in muscle extract shows a steady liberation of inorganic phosphate and lactic acid, as Embden [1914] first showed. Glycogen, in the type of extract used here, shows the same steady rate of lactic acid formation, but in this case the accompanying phosphate changes are of three types, depending on the glycogen concentration and also on the nature of the extract. This classification is based on the results of investigations with about a hundred different extracts. These three types are indicated in Fig. 1: the graphs show the changes in inorganic phosphate plotted against the time of incubation.

Type 1. In this type there is no ester accumulation even with concentrations of glycogen as great as 0·8 %. The phosphorus concentration remains practically the same until all the glycogen has been used up, after which the phosphate increases. This increase is from pre-existing lactacidogen in the extract.

Type 2. In this type, no ester accumulation takes place during the first 30 to 40 minutes of the incubation, but thereafter an extensive and rapid accumulation takes place between 40 and 90 minutes from the start. This type is often produced by concentrations of glycogen of the order of 1·0 % in extracts that give a "type 1" curve with about 0·5 % glycogen.

Type 3. In this type the ester accumulation is intense at the very start, is completed in most cases in the first 10 to 20 minutes of the incubation period and is followed by a second period of breakdown of the ester.

The inorganic phosphate liberated in an extract of the type used here, to which no carbohydrate has been added, ranges from 25 to 35 mg. per 100 cc. This process is completed in about 60 minutes. This would indicate a certain concentration of lactacidogen because the liberation of the phosphate is always accompanied by lactic acid formation and the two processes slow down and stop together. It seemed not at all unlikely that the difference between a "type 3" and a "type 1" extract, for example, lay in the fact that the latter

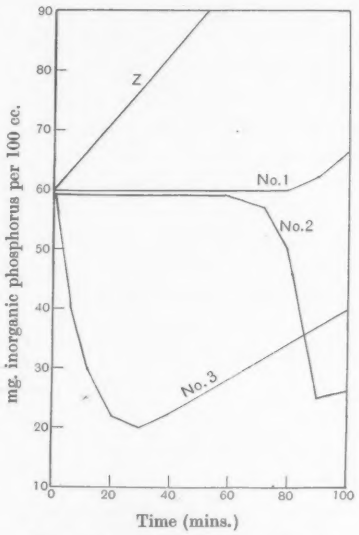

Fig. 1. Inorganic phosphorus changes.

Z. Plain sodium hexosediphosphate.
No. 1 type with glycogen.
No. 2 „ „
No. 3 „ „

type contained as a rule more lactacidogen than the former. The "phosphagen" content is likewise variable, but the present communication excludes investigations on the possible rôle of this labile phosphorus compound in this connection. This has been made the subject of investigation for a time in this laboratory and the results will be recorded in a subsequent communication. Considered here is solely the possible influence of the acid-stable lactacidogen and the related substance hexosediphosphate.

It appeared quite possible that if the lactacidogen were broken down at the same rate as the ester was being formed from the glycogen, the phos-

phorus liberated from the lactacidogen could be passed on to the glycogen (or to whatever breakdown product is converted into the ester) at a rate sufficient to keep the ester formation going at its maximum rate. This would spare, so to speak, the inorganic phosphate of the extract from taking part in this process, the concentration of inorganic phosphate would not change and the flat "type 1" curve would be produced. If the lactacidogen were not present to a great extent at the start, then the phosphorus liberated might not be set free at a rate great enough to keep the rate of ester formation at its maximum, and a call would then be made on the inorganic phosphate of the extract and thus a "type 3" curve might result. It was thought that, if, in the case of an extract yielding a "type 3" curve with glycogen, sodium hexosediphosphate were added along with the glycogen, the liberation of phosphate from this ester might be sufficiently rapid to balance the removal of phosphate by the esterification process, and thus a "type 3" curve might be converted into a flat "type 1" curve. The assumption made throughout is that lactacidogen or hexosediphosphate breakdown takes place in the presence of glycogen and simultaneously with the formation of ester from the latter. The phosphate curve is thus assumed to represent the balance between phosphoric ester breakdown and synthesis. As the experimental results will show, the production of a "type 1" curve is not conditioned by a high initial concentration of phosphoric ester; on the contrary the presence of a phosphoric ester along with glycogen either has no effect or leads to a decided accumulation of ester. Contrary to expectation a "type 1" curve is converted into a "type 3" curve.

Experimental procedure.

The extracts were prepared in exactly the same manner as those described in the first paper of this series, and when any special treatment is given it is stated. Two preparations of hexosediphosphate were used. One was prepared in this laboratory according to a private communication from Prof. Harden, using a dried yeast from the local brewery. The other preparation was a gift to the department from Bayer Products, Ltd. Both preparations yielded the same results.

The glycogen was added either as a 20 % or a 10 % suspension made up in boiling water. The sodium hexosediphosphate was added in the case of the Bayer product as a 20 % solution; in the case of the author's preparation, the strength of the stock solution, concentrated by distillation under reduced pressure, was determined from the difference between the inorganic phosphorus and the total phosphorus as estimated by the nitric acid method of wet ashing. In the case of the plain glycogen controls, water was added equivalent to the volume of the zymophosphate. The addition of these solutions and the method of sampling were as described in the previous paper.

Experimental results.

In Exp. 217, a typical case, the phosphorus changes are represented in Fig. 2. All inorganic phosphorus concentrations are expressed as mg. per 100 cc. extract. Lactic acid estimations were not made in several of these preliminary experiments.

In Exp. 218 (Fig. 3) the presence of the zymophosphate shifted the period of maximum ester accumulation to an earlier point in the incubation. The higher concentration of zymophosphate is more effective than the lower.

In Exp. 219 the following were tried. Glycogen 0·33 % alone and with 1·2 % zymophosphate; glycogen 0·66 % alone and with 0·6 % and 1·2 % zymophosphate; glycogen 1·3 % alone and with these same two concentrations of zymophosphate. In those cases where the glycogen concentration

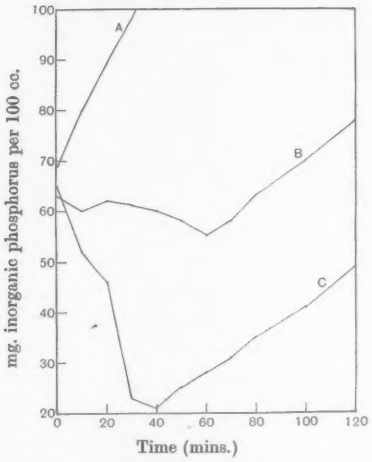

Fig. 2. Exp. 217.
A. Zymophosphate 1·0 % alone.
B. Glycogen 0·65 % alone.
C. Glycogen 0·65 % with zymophosphate 1·0 %.

was 0·33 % or 0·66 %, none of the zymophosphate concentrations was effective. In the case of 0·33 % glycogen with 1·2 % zymophosphate, there was more phosphate liberated in the later stages of the incubation than with glycogen alone, indicating at this stage that, after all the glycogen had been broken down, the zymophosphate was hydrolysed. The results with the 1·3 % glycogen are shown in Fig. 4.

In Exp. 224, one-half of the extract was acidified to about p_H 6·5 by HCl, the molar equivalent of NaCl being added to the other half which was adjusted to p_H 7·5. The results are shown in Fig. 5. The acid extract gave the better result; the glycogen curve is a typical "type 1" curve and the effect of the zymophosphate is very decided.

In the next set of experiments the lactic acid formation was followed in addition to the changes in inorganic phosphorus. The lactic acid was estimated by the method mentioned in the preceding paper. The results of Exp. 238 are not given in detail. The phosphorus changes in a mixture of 1·5 % zymophosphate and 0·7 % glycogen were very similar to those in the 0·7 % glycogen alone. Nevertheless it was noticed that the total lactic acid formed with glycogen alone in 90 minutes was 167 mg. per 100 cc. extract and in the glycogen-zymophosphate mixture only 130 mg., a decrease of 22 %.

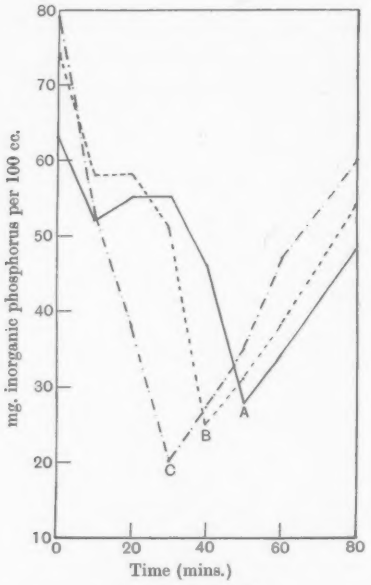

Fig. 3. Exp. 218.

A. Glycogen 0·6 % alone.
B. ,, 0·6 % with zymophosphate 0·6 %.
C. ,, 0·6 % ,, 1·2 %.

In Exp. 240 glycogen 0·9 % alone and in conjunction with 1·0 % zymophosphate was investigated. There was a slight "extra ester accumulation" in this case associated with a 30 % decrease in the lactic acid production. The lactic acid produced in the plain glycogen was 130 mg. and in the glycogen plus zymophosphate mixture only 90 mg. on incubation for 90 minutes.

Exp. 241 was, however, a good case. Two concentrations of glycogen were tried with 1·0 % zymophosphate. In the case of 0·4 % glycogen, the zymophosphate did produce extra ester equivalent to 26 mg. of phosphorus. At the end of 60 minutes, the lactic acid increase in the plain 0·4 % glycogen was 226 mg. and in the corresponding glycogen-zymophosphate mixture only 167 mg., a decrease of 26 %.

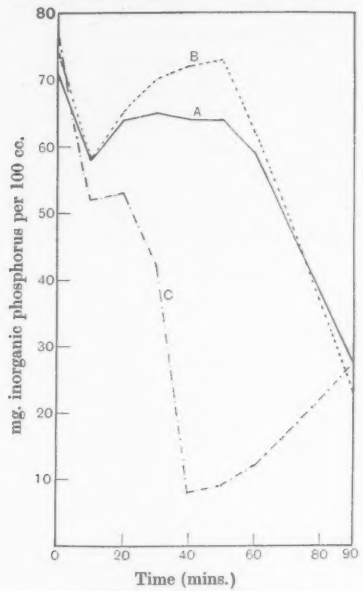

Fig. 4. Exp. 219.

A. Glycogen alone 1·3 %.
B. „ 1·3 % with zymophosphate 0·6 %.
C. „ 1·3 % „ 1·2 %.

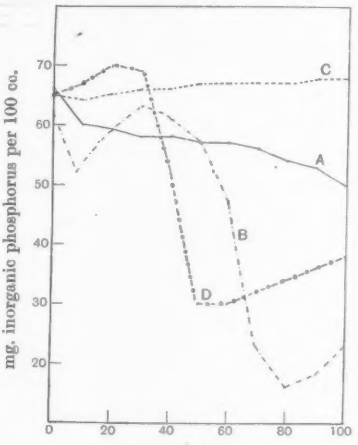

Fig. 5. Exp. 224.

A. Glycogen 0·9 %, p_H 7·5.
B. „ 0·9 % with zymophosphate 1·5 %, p_H 7·5.
C. „ 0·9 %, p_H 6·5.
D. „ 0·9 % with zymophosphate 1·5 %, p_H 6·5.

The results with 1·0 % glycogen alone and with 1·0 % zymophosphate with the extract of Exp. 241, are shown in the graphs of Figs. 6 and 7. It will be seen that the inhibition of the lactic acid formation by the zymophosphate comes decidedly into evidence after the first 10 minutes of the incubation and when the "extra ester accumulation" effect begins to appear. In this case the 1·0 % glycogen alone showed at the end of 90 minutes a production of 439 mg. lactic acid, the glycogen-zymophosphate mixture only 171 mg., a decrease in this case of 61 %. Although, in this case, the

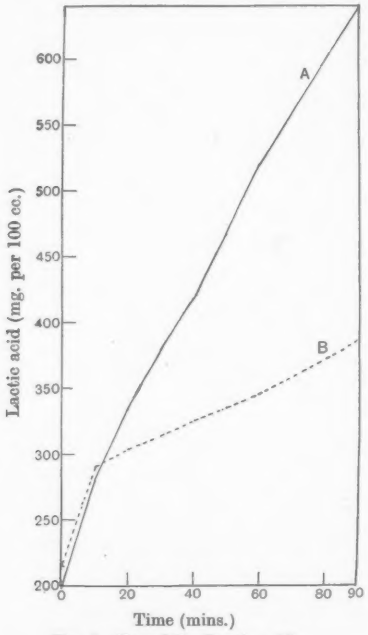

Fig. 6. Exp. 241. Lactic acid.
A. Glycogen 1·0 % alone.
B. Glycogen 1·0 % with zymophosphate 1·0 %.

inhibition of the lactic acid formation is related to the "extra ester accumulation," inhibition of the lactic acid production is not apparently always accompanied by any decided effect of the zymophosphate on the phosphorus changes in the controls.

In addition to the above observations the following was noted. Using 3 cc. of the trichloroacetic filtrates which were used for the inorganic phosphate estimations and adding 0·3 cc. of $N/50$ iodine, the reddish brown colour of the glycogen iodine compound is produced. By comparing in a comparator the colour of the corresponding samples of plain glycogen and of glycogen-zymophosphate mixture, a decided diminution in the strength of the colour

is readily detected in those cases where "extra ester accumulation" is produced to a decided extent by the zymophosphate. In addition, therefore, to the inhibition of the lactic acid formation, there occurs this phenomenon of more rapid loss of glycogen.

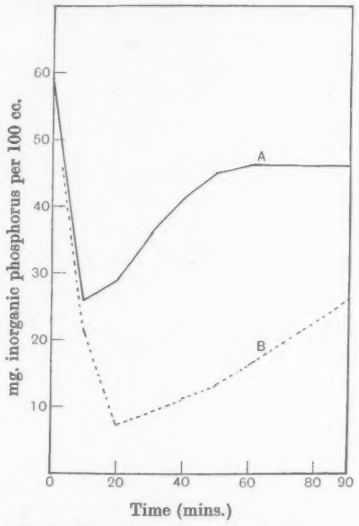

Fig. 7. Exp. 241. Inorganic phosphorus.
A. Glycogen 1·0 % alone.
B. Glycogen 1·0 % with zymophosphate 1·0 %.

Summary.

With the type of extract used the following has been noted. In a mixture of zymophosphate and glycogen in certain concentrations, there is less lactic acid produced than in the corresponding glycogen concentration in the absence of zymophosphate. This is associated, not invariably but very frequently, with an increased accumulation of phosphoric ester in the glycogen-zymophosphate mixture as compared with the corresponding glycogen experiment. This "extra ester accumulation," due to the presence of zymophosphate, is also associated with a more rapid loss of glycogen in addition to the abovementioned inhibition of the lactic acid formation.

In concluding the author wishes to express his indebtedness to Bayer Products, Ltd., for a gift of 100 g. of pure sodium hexosediphosphate.

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CXI. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

III. GLYCOLYSIS IN STERILE CELL-FREE EXTRACTS OF MUSCLE.

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(*Received May 4th, 1928.*)

Since the time of Claude Bernard, much investigation has been done on the subject of the "glycolytic enzyme" of blood and of muscle. The results of these investigations are very varied and even contradictory. One aspect of the subject, however, strikes one as worthy of further consideration. In the past, very few of the muscle extracts were bacteriologically sterile. It is certainly true that antiseptics were generally employed; but while on the one hand a negative result under these conditions proves nothing conclusive, since the antiseptic may be an inhibitor of the enzyme, on the other hand, a positive result, especially with an incubation period of more than three or four hours, makes one doubt the efficiency of the antiseptic. Whether a given antiseptic is an inhibitor of enzyme activity in the concentration required to prevent effectively the growth of microorganisms, or is an indifferent substance, cannot be demonstrated beyond doubt until the effect of its presence has been studied in an extract made sterile by some other method. If the problem of sterility is to be overlooked the period of incubation must not exceed three hours, and if in that period the loss of glucose is small, the results are not very convincing. The solution of this question lies in preparing extracts that are sterile, free from antiseptics and with a glycolytic activity of such magnitude that the loss of glucose is rapid. Such extracts have been prepared in this laboratory. The method consisted in filtering the muscle extract, previously frozen to a thick cream, through Berkefeld filter candles and then, at the end of the incubation, testing both for aerobic and anaerobic organisms by inoculation of a sample of the extract into Douglas broth incubated both aerobically and anaerobically.

This method of producing sterility was first applied to muscle extract by Brunton and Rhodes [1901]. They filtered an extract of sheep muscle through Pasteur-Chamberland filters: on keeping the filtrates in bottles plugged with cotton wool for several weeks, no bacterial growth was observed. The concentration of glucose fell from 1·5 to 0·75 % in 48 hours and this result was confirmed several times.

Since the experiments described in this paper were performed, Meyerhof [1926] also mentions that he was able to observe lactic acid formation from glycogen in extracts of muscle filtered through Berkefeld filters. His results were similar to those described here in that the first portion of the filtrate is inactive; but the experiments below were conducted with the intention of detecting destruction of glucose, not the breakdown of glycogen. It is in this sense that the term glycolysis is used here. In the investigator's experience it is more difficult to get an extract showing active glycolysis than one showing rapid lactic acid formation from glycogen. Glycolytic activity in this sense

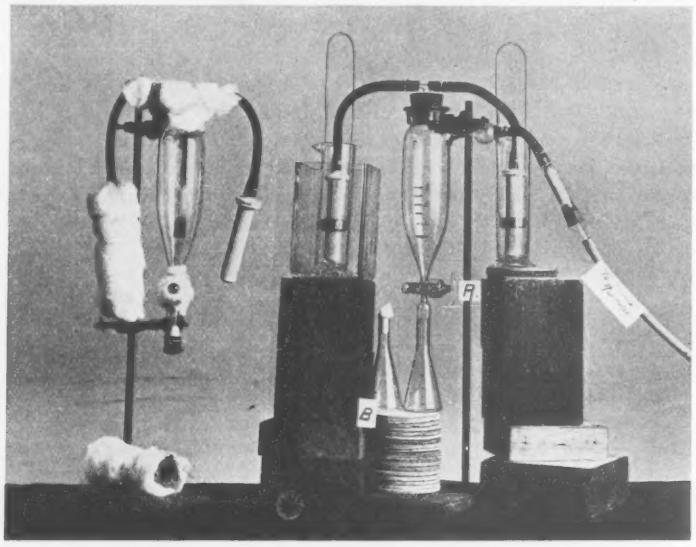

Fig. 1.

is a very uncertain phenomenon for some reason as yet obscure. Using certain precautions, however, it was possible in a great many cases, although not without many failures, to prepare sterile extracts of perfused rabbit and cat muscle, free from any intact cell structures, with a glycolytic activity of such a degree that the existence of the "glycolytic complex" in an active condition in cell-free extracts of muscle can no longer be considered doubtful.

Preparation and filtration of the extracts.

The animals were killed by a sharp blow on the head and immediately perfused with Ringer solution. The treatment thereafter was as is described in the first paper of this series, only in the cases described here the extracting

solution was one containing sodium bicarbonate and disodium hydrogen phosphate (hydrated) both to the extent of 0·5 %.

For the filtration the apparatus shown in Fig. 1 was employed. The two candles are joined by a T-piece fitted into a rubber stopper inserted into the top of the large collecting vessel known as a "Massen." The quickest filtration was obtained by reducing the pressure inside the "Massen" to about 100 mm. Hg and at the same time continually clearing the surface of the candles by the scrubbers as shown in the figure. These scrubbers consist of a ring of brass slightly wider than the diameter of the candle and lined on the inside with a strip of rubber pay-mat. By moving the scrubber up and down over the surface of the candle, the raised points of rubber clean the surface free of the slime that accumulates and chokes the filter.

For convenience the "Massen" was graduated to show every 50 cc. up to 300 cc. The collected filtrate was run out through the tap (*A*) into specially made conical flasks (*B*). The outlet tube from (*A*) is enclosed in a hood of wider bore; the conical flasks were therefore made with a long neck without any lip and of such a diameter that they could be pushed up inside the hood. The transference of the filtrate from the "Massen" to the conical flasks could thus be done without risk of infection, if the usual bacteriological precautions were taken.

The "Massen" and attached filters were sterilised by autoclaving at 120° for 20 minutes. The apparatus ready for sterilisation is shown on the left of Fig. 1. The tap (*A*) was tightly wrapped up in a pad of cotton wool, because when the suction is applied there often occurs at this tap a leak of air into the "Massen" in spite of the fact that the tap has been well greased by a mixture of white wax and lanolin. Since rubber shrinks on heating, the stopper with the T-piece tends to become loose and consequently was likewise covered up with a thick layer of cotton wool; it could thus be reinserted when the apparatus was cold without undue risk. When the required volume of the extract had been filtered, the glucose was introduced into the "Massen" via the tap (*A*). The tap bore was first cleared free of wax by a hot wire, the autoclaved glucose solution was introduced through the bore of the tap by means of a sterilised glass tube drawn out to a long thin point.

After shaking up, a few cc. were run out to wash out any glucose that might have adhered to the sides of the outlet tube. The extract was then measured out into the conical flasks, 25 cc. in each. One flask was taken for the initial values of glucose; the proteins were precipitated by tungstic acid and the glucose estimated by the method of Schaffer and Hartmann [1921]. The other samples were tested for sterility and analysed at the end of the incubation. For the detection of bacteria, two tubes of Douglas broth were each inoculated with about 1 cc. of the extract. One was incubated in the usual manner for aerobes. In the other the cotton wool plug was flamed and pushed in. The tube was fitted with a rubber stopper carrying a glass tube drawn out to a constriction ready for sealing: the air was sucked out and re-

placed by purified hydrogen; this alternate evacuating and refilling with hydrogen was done five times and lastly the tube was sealed when evacuated. This tube was incubated for the detection of anaerobes.

The difficulty of producing sterile filtrates was overcome after several failures but it was soon realised that all Berkefeld filters do not necessarily give sterile filtrates and unless one uses candles previously tested for their ability to hold back bacteria (in this case a peptone culture of *B. pyocyaneus* was used and the filtrate plated on agar), sterility is somewhat uncertain, and this entailed a great waste of effort. This difficulty was connected with the even greater one of producing extracts that were glycolytically active to a decided extent. Again after many failures the following details of technique had to be adopted.

The reaction of the extract must be on the alkaline side of p_H 7·0; on the acid side of this the proteins do not pass through the pores of the filter, the candles choke very early in the filtration and the filtrates are inactive. This was the experience of Mudd [1922].

The extracts must be well frozen to a good stiff cream before the filtration is started so that the extract enters the filters in a concentrated condition by the freezing out of the water. Even with these precautions the best results were invariably obtained by using new, dry, but autoclaved filters. There was always however the attendant risk of infection under these conditions as the candles had not been previously tested. It was not possible to use new candles on every occasion. The most effective method of clearing an old filter was to fill it with 0·2 *N* sulphuric acid and to autoclave it in this condition. This produces a rapidly filtering candle, and in many cases this treatment could be repeated four or five times without the structure being destroyed or the ability to hold back bacteria being lost. Such filters, washed free of acid by bicarbonate and sucked "dry" on the aspirator, lose weight to the extent of 30 g. if heated at 100° until of constant weight. When using filters in this condition, *i.e.* with the kieselguhr skeleton damp, it was found that the first 100 cc. of the filtrate could be rejected because there was little or no glycolytic activity in this fraction; but the next 100 to 150 cc. usually showed glycolytic activity. With new, dry filters the whole of the filtrate could be used. The process of drying by heating to about 100° rapidly leads to the crumbling of the filter and for that reason it was given up; but, if the early portions of the filtrate were rejected, these old filters could be used again and again.

Experimental results.

Although no lactic acid estimations were made in this series given below, later work has indicated that the end product is lactic acid, a fact now well established. Never, in any single instance (and 34 cases were investigated for this) was any evidence found of the formation of a more complex carbohydrate (disaccharide) as suggested by Levene and Meyer [1912]. The glucose concentrations are expressed as mg. per 100 cc. extract.

Table of results.

No. of exp.	Glucose Start	Glucose End	Glucose Loss	Time hrs.	Remarks
50 *B*	135	22	113	24	Tame rabbit
54 *C*	112	Trace	111	24	„
57 *B*	149	„	148	24	„
59 *B*	157	25	132	24	„
60 *C*	136	Trace	135	24	„
73 *B*	133	„	132	4	„
77 *B*	154	12	142	4	Cat
79	169	169	0	3	1st 50 cc. Tame rabbit
	182	173	9	3	2nd 50 „
	173	98	75	3	3rd 50 „ Damp filters
	187	55	132	3	4th 50 „
82	147	71	76	2	1st 50 cc. Tame rabbit
	146	19	127	2	2nd 50 „
	155	24	131	2	3rd 50 „ New dry filters
	146	88	58	2	4th 50 „
	152	129	23	2	5th 50 „
83	254	243	11	2	1st 50 cc. Tame rabbit
	285	243	42	2	2nd 50 „
	268	177	91	2	3rd 50 „
	258	197	61	2	4th 50 „ Damp filters
	251	120	131	2	5th 50 „
86	308	113	195	3	1st 100 cc. Tame rabbit
	332	103	229	3	2nd 100 „
87	512	319	132	3	1st 100 cc. Tame rabbit
89	351	21	330	8	Tame rabbit
91	641	402	239	3	„
93	574	407	167	3	Cat
95	332	152	180	2	„
96	336	119	217	3	Two tame rabbits
97	355	179	176	3	Cat
99	330	174	156	2	1st 50 cc. Two tame rabbits
	345	170	175	2	2nd 50 „
	327	122	205	2	3rd 50 „
	335	216	119	2	4th 50 „ New dry filters
	343	279	64	2	5th 50 „
100	345	129	216	2	Two tame rabbits
102	308	233	75	1	1st 75 cc. Cat
	305	152	153	2	2nd 75 „ New dry filters
	291	120	171	2	3rd 75 „
	291	38	253	2	„ „ plus 0·1 g. of $NaHCO_3$

Quoted above are 21 cases out of a total of 102 extracts filtered. The 81 cases not recorded include both those that were infected and those that showed little or no loss of glucose. The latter are interesting although not of direct value in a positive sense. Many of the early failures were due to the use of an unsuitable extracting solution. The results showed that a mixture of sodium or potassium bicarbonate with the chlorides or dibasic phosphates of those metals was quite suitable if the salts were used in concentrations not greater than about 1 %. A solution of 5 % $Na_2HPO_4, 12H_2O$ gave an extract that coagulated during the filtration. The extraction of the muscle proteins was very extensive with this solution but that very fact rendered it highly unsuitable for filtration purposes.

Another source of failure, which was not appreciated until experiments had been done with unfiltered extracts, is that tame rabbits are not, for some peculiar reason, the best animals for the production of very glycolytic extracts.

During the course of the author's investigations on glycolysis, over 200 different extracts have been examined and the best animals for this type of investigation have been found to be either wild rabbits or cats. Even with these animals one occasionally encounters extracts of apparently normal individuals that show only a slight glycolytic activity, no matter how rigorously a standardised method of preparation is employed. In the case of tame rabbits, extracts prepared in the above manner show good glycolysis in only about 50 % of the cases. In this respect lactic acid formation from glucose must be distinguished from lactic acid formation from glycogen, the latter unlike the former takes place at a fairly uniform rate in all freshly prepared extracts. This is still under investigation.

Discussion.

The above results indicate that the glycolytic enzyme (or enzymes) can be demonstrated by the destruction of added glucose in filtrates from Berkefeld filters. No activator of any description, such as the pancreatic extract employed by Cohnheim [1903; 1904, 1, 2; 1907], was added. More important still, no intact cell structures were present, and the condition put forward by Fletcher [1911], that glycolysis can only take place if the muscle structure is preserved, is not indicated here at all. If the above type of extract be centrifuged in a Sharples supercentrifuge, a clear transparent solution is produced, similar to the filtrates from the Berkefeld filters, but of course not sterile. The removal, in this manner, of the muscle debris that passes through the mesh of the muslin used in the hand-press, has absolutely no effect on the glycolytic activity. The muscle enzymes are in solution in the same sense as the muscle proteins, although removed from the internal environment of the muscle fibres.

The question of sterility is not, in the author's opinion, an important one for the following reason. An examination of the literature of this subject gives one the impression that an incubation period of at least 24 hours is necessary before a decided loss of glucose can be detected; but an extensive examination of many extracts at intervals of 10 minutes over a period up to 2 hours, indicated that the greatest rate of destruction of glucose takes place in the first 10 minutes of the incubation. In extracts of cat muscle the loss of glucose in the first 10 minutes has been observed to be as high as 78 mg. per 100 cc. extract: with wild rabbit muscle it has been as high as 95 mg. This high rate lasts for about 40 minutes; consequently the period of incubation need not exceed, at the longest, 2 hours, and even 90 minutes is quite sufficient. In that time the effect of bacterial growth is not evidenced. The many cases of unfiltered extracts, prepared with no precautions against infection and showing no loss of glucose at the end of a 2 hours' incubation, indicate that it is the muscle enzymes and not bacteria that give rise to lactic acid in the early period of the incubation, and it is in that early period that the most important and interesting phase of lactic acid formation takes place.

SUMMARY.

Glycolysis has been observed in extracts of perfused muscle after filtration through Berkefeld filters. The results quoted are with those filtrates that were bacteriologically sterile. The original observation of Brunton and Rhodes on glycolysis in similar filtrates has been confirmed.

In concluding, the author wishes to express his gratitude to Prof. E. Waymouth Reid, F.R.S., who suggested and supervised this research; likewise his thanks to Mr W. Milne for taking charge of the sterilising and care of the apparatus, and also to Prof. W. J. Tulloch, for his advice on the bacteriological side of the problem.

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